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**EMERGING CONTAMINANTS IN AGRICULTURAL
ECOSYSTEMS: IMPACT OF SELECTED
PHARMACEUTICALS ON WATER AND SOIL ECOLOGY
AND PRACTICAL IMPLICATIONS**

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ABSTRACT.....	1
1. INTRODUCTION	4
1.1 Emerging contaminants	4
1.2 Fate of contaminants in the agro-ecosystem	6
1.3 Why are emerging contaminants a concern?	8
1.4 Microbial ecology approach	10
1.5 Legislation in the European Union	14
1.6 Bioremediation.....	16
2. AIM OF THE THESIS AND SELECTED PHARMACEUTICALS	18
2.1 Fate and removal of the antiviral drug oseltamivir (Tamiflu) in superficial water.....	19
2.2 Fate and removal of common use antibiotics in WWTP	21
2.3 Fate and removal of veterinary pharmaceuticals in soil	22
3. MATERIALS AND METHODS	24
3.1 CHAPTER 1 – The antiviral drug Tamiflu.....	24
3.1.1 Chemical analysis.....	24
<i>a. Degradation analysis by HPLC</i>	<i>24</i>
<i>b. Degradation analysis by LC-ESI-MS/MS</i>	<i>24</i>
<i>c. Mineralization analysis</i>	<i>25</i>
<i>d. Bioavailability and sorption isotherms</i>	<i>25</i>
3.1.2 Microbial analysis	26
Bacterial community structure	27
<i>e. Bacterial abundance by direct count (DAPI).....</i>	<i>27</i>
<i>f. Bacterial phylogenetic composition by Fluorescence In Situ Hybridization (FISH)</i>	<i>27</i>
<i>g. Microbial community structure by Amplified Ribosomal DNA Restriction Analysis (ARDRA)</i>	<i>29</i>
<i>h. Microbial community structure by Denaturing Gradient Gel Electrophoresis (DGGE).....</i>	<i>30</i>
Bacterial community function.....	31
<i>i. Bacterial viability by direct count.....</i>	<i>31</i>
<i>j. Bacterial metabolic potential.....</i>	<i>31</i>
<i>k. Nitrifying bacteria quantification by quantitative PCR (qPCR and qRT-PCR)</i>	<i>32</i>
<i>l. Bacterial isolates and bioremediation tests</i>	<i>34</i>
3.1.3 Study cases in different water ecosystems	36
3.1.3.1 Irrigation canal in Emilia Romagna	36
3.1.3.2 River Po and Venice Lagoon	37
3.1.3.3 Lake Biwa and River Furukawa.....	40

3.1.3.4 Wastewater Treatment Plant of Bologna	41
3.2 CHAPTER 2 – Fate and removal of common use antibiotics in WWTP	42
3.2.1 Experimental site, sampling and microcosm set up	42
3.2.2 Chemical analysis	43
3.2.3 Microbial analysis	43
3.3 CHAPTER 3 – Veterinary Pharmaceuticals	45
3.3.1 Experimental site, sampling and microcosm set up	45
3.3.2 Microbial analysis	45
4. RESULTS AND DISCUSSION	48
4.1 CHAPTER 1 – The antiviral drug Tamiflu	48
4.1.1 Irrigation canal in Emilia Romagna	48
4.1.2 River Po and Venice Lagoon	55
4.1.3 Lake Biwa and River Furukawa	61
4.1.4 Wastewater Treatment Plant of Bologna	69
4.2 CHAPTER 2 – Common use antibiotics in WWTP	74
4.3 CHAPTER 3 – Veterinary pharmaceuticals	77
5. CONCLUSIONS	80
5.1 CHAPTER 1	80
5.2 CHAPTER 2	81
5.3 CHAPTER 3	81
REFERENCES	82
ACKNOWLEDGEMENTS	94

ABSTRACT

Pharmaceuticals are useful tools to prevent and treat human and animal diseases. Following administration, a significant fraction of pharmaceuticals is excreted unaltered into faeces and urine and may enter the aquatic ecosystem and agricultural soil through irrigation with recycled water, constituting a significant source of emerging contaminants into the environment. Understanding major factors influencing their environmental fate is consequently needed to value the risk, reduce contamination, and set up bioremediation technologies.

The antiviral drug Tamiflu (oseltamivir carboxylate, OC) has received recent attention due to the potential use as a first line defence against H5N1 and H1N1 influenza viruses. Research has shown that OC is not removed during conventional wastewater treatments, thus having the potential to enter surface water bodies. A series of laboratory experiments investigated the fate and the removal of OC in surface water systems in Italy and Japan and in a municipal wastewater treatment plant.

A preliminary laboratory study investigated the persistence of the active antiviral drug in water samples from an irrigation canal in northern Italy (Canale Emiliano Romagnolo). After an initial rapid decrease, OC concentration slowly decreased during the remaining incubation period. Approximately 65% of the initial OC amount remained in water at the end of the 36-day incubation period. A negligible amount of OC was lost both from sterilized water and from sterilized water/sediment samples, suggesting a significant role of microbial degradation. Stimulating microbial processes by the addition of sediments resulted in reduced OC persistence. Presence of OC ($1.5 \mu\text{g mL}^{-1}$) did not significantly affect the metabolic potential of the water microbial population, that was estimated by glyphosate and metolachlor mineralization. In contrast, OC caused an initial transient decrease in the size of the indigenous microbial population of water samples.

A second laboratory study focused on basic processes governing the environmental fate of OC in surface water from two contrasting aquatic ecosystems of northern Italy, the River Po and the Venice Lagoon. Results of this study confirmed the potential of OC to persist in surface water. However, the

addition of 5% of sediments resulted in rapid OC degradation. The estimated half-life of OC in water/sediment of the River Po was 15 days. After three weeks of incubation at 20 °C, more than 8% of ^{14}C -OC evolved as $^{14}\text{CO}_2$ from water/sediment samples of the River Po and Venice Lagoon. OC was moderately retained onto coarse sediments from the two sites. In water/sediment samples of the River Po and Venice Lagoon treated with ^{14}C -OC, more than 30% of the ^{14}C -residues remained water-extractable after three weeks of incubation. The low affinity of OC to sediments suggests that the presence of sediments would not reduce its bioavailability to microbial degradation.

Another series of laboratory experiments investigated the fate and the removal of OC in two surface water ecosystems of Japan and in the municipal wastewater treatment plant of the city of Bologna, in Northern Italy. The persistence of OC in surface water ranged from non-detectable degradation to a half-life of 53 days. After 40 days, less than 3% of radiolabeled OC evolved as $^{14}\text{CO}_2$. The presence of sediments (5%) led to a significant increase of OC degradation and of mineralization rates. A more intense mineralization was observed in samples of the wastewater treatment plant when applying a long incubation period (40 days). More precisely, 76% and 37% of the initial radioactivity applied as ^{14}C -OC was recovered as $^{14}\text{CO}_2$ from samples of the biological tank and effluent water, respectively. Two bacterial strains growing on OC as sole carbon source were isolated and used for its removal from synthetic medium and environmental samples, including surface water and wastewater. Inoculation of water and wastewater samples with the two OC-degrading strains showed that mineralization of OC was significantly higher in both inoculated water and wastewater, than in uninoculated controls. Denaturing gradient gel electrophoresis and quantitative PCR analysis showed that OC would not affect the microbial population of surface water and wastewater.

The capacity of the ligninolytic fungus *Phanerochaete chrysosporium* to degrade a wide variety of environmentally persistent xenobiotics has been largely reported in literature. In a series of laboratory experiments, the efficiency of a formulation using *P. chrysosporium* was evaluated for the removal of selected pharmaceuticals from wastewater samples. Addition of the fungus to samples of the wastewater treatment plant of Bologna significantly increased ($P < 0.05$) the removal of OC and three antibiotics, erythromycin, sulfamethoxazole, and

ciprofloxacin. Similar effects were also observed in effluent water. OC was the most persistent of the four pharmaceuticals. After 30 days of incubation, approximately two times more OC was removed in bioremediated samples than in controls. The highest removal efficiency of the formulation was observed with the antibiotic ciprofloxacin.

The studies included environmental aspects of soil contamination with two emerging veterinary contaminants, such as doramectin and oxibendazole, which are common parasitic treatments in cattle farms.

1. INTRODUCTION

1.1 Emerging contaminants

During the past decade, the increasing introduction in the market of new chemicals, and the development of more accurate analytical methods, added a variety of new environmental ‘emerging’ contaminants to the already large array of pollutants. Emerging contaminants are defined as any synthetic or naturally occurring chemical that is not commonly monitored in the environment, though having the potential to enter soil and aquatic ecosystems, causing known or suspected adverse ecological and/or human health effects (USGS, 2009).

Recent concern are receiving chemicals that have been detected in varied water sources, such as antibiotics, anti-depressants, tranquilizers, endocrine disrupting chemicals, personal care products, illicit drugs, fluorinated compounds and nanomaterials. Although present in the environment at low concentrations, in the range of ng L^{-1} , most of these ‘micropollutants’ raise considerable toxicological concern, particularly if present as components of complex mixtures (Schwarzenbach et al., 2006). Emerging contaminants can enter the environment by a variety of sources, such as sewage treatment plants (STPs), runoff from agricultural land uses, aquaculture and livestock farming, industrial wastes and hospital effluents (Figure 1).

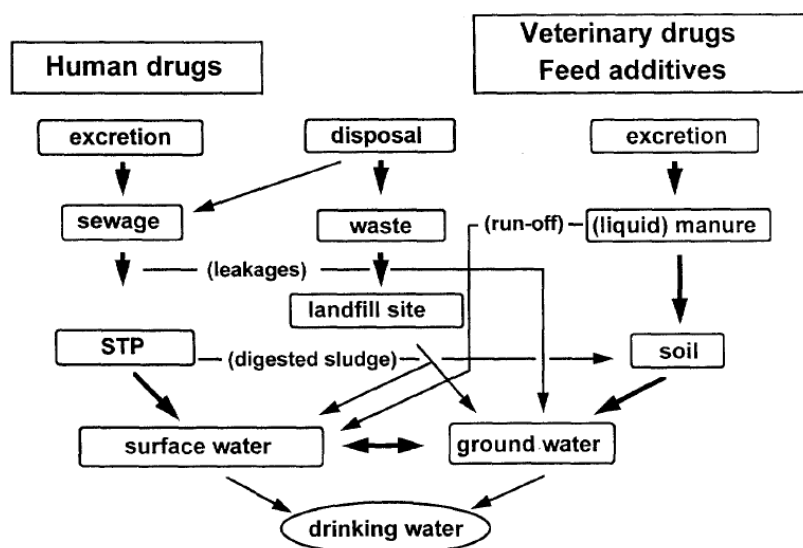


Figure 1 – Schematic representation of the fate of human and veterinary drugs, after application (Ternes, 1998).

The occurrence of pharmaceuticals in the environment is a recent issue. Research on this topic started in the 1990s, when Ternes, a German chemist, investigated the environmental fate of a group of prescribed medicines after excretion (Ternes, 1998). These were the first results of monitoring studies of pharmaceutical measurements in local STPs and rivers.

Thousands of different pharmaceutically active compounds are actually used in high quantities to treat or to prevent diseases (Kümmerer et al., 2009a; Bottoni et al., 2010). Following therapeutic administration, a great percentage of pharmaceuticals is excreted in urine and faeces (Carlsson et al., 2006) as parent compound and/or metabolites and enters the sewage treatment system, where they are often only partially removed (Halling-Sørensen et al., 1998). In a study conducted in Italy, Zuccato et al. (2005) detected a variety of pharmaceuticals in STPs and in the River Po (Table 1). Some of the detected molecules are rapidly degraded (i.e. ibuprofen, sulfamethoxazole), while others are reported to be persistent in surface water (i.e. atenolol, carbamazepine, ciprofloxacin, erythromycin, ofloxacin). Degradation of xenobiotics in the aquatic ecosystem depends on a variety of factors, including compound properties and environmental factors (Lu et al., 2006).

	Type of drug	STP (ng L ⁻¹)	River Po (ng L ⁻¹)
Ofloxacin	chemotherapeutic antibiotic	600.0	33.1
Atenolol	used in cardiovascular diseases	466.0	17.2
Hydrochlorothiazide	diuretic	439.1	4.6
Carbamazepine	used to prevent and control seizures	291.1	23.1
Ciprofloxacin	chemotherapeutic antibiotic	251.0	Nd
Sulfamethoxazole	sulfonamide bacteriostatic antibiotic	127.2	Nd
Ibuprofen	anti-inflammatory	121.2	13.0
Erythromycin	wide spectrum antibiotic	47.4	3.2

Table 1 – Pharmaceutical concentrations detected in urban sewage treatment plants (STPs) (median of nine STPs) and in River Po (median value of seven sampling sites) (Zuccato et al., 2005).

Sewage treatments plant are usually designed to remove or to reduce the concentrations of microbial pathogens and loads of organic bulk, but they are not specifically designed to remove pharmaceuticals or biologically active substances. Furthermore, when a chemical is not detectable, it only means that the parent

compound has been removed from the compartment of interest, and eventually it has only been transformed in a degradation product. Degradation of a parent molecule consists in reactions of oxidation, reduction or hydrolysis, and its transformation products are often more reactive and sometimes more toxic than the parent drug. By changing the physico-chemical behaviour of the substance, degradation processes can modify its water solubility (usually an increase of water solubility is expected) with respect to the parent compounds (Halling-Sørensen et al., 1998). Present level of knowledge about the degradation pathways in STPs is not always exhaustive, and wastewater is one of the major sources of micropollutants in the environment (Schwarzenbach et al., 2006).

The two major mechanisms involved in the removal of substances from the incoming waste stream in STPs are the following: microbial degradation and sorption onto solid particles. The removal rate of pharmaceuticals in STPs is affected by several factors, including their physico-chemical properties, the adopted treatment process, hydraulic and sludge retention time, environmental parameters, and properties of the influent (O'Brien and Dietrich, 2004). Sludge retention time has been considered as one of the most important process parameters. For highly polar substances, as most pharmaceuticals are, the most important removal process is biological transformation or mineralization by microorganisms; if the residence time is too short, it will not implement an efficient biodegradation. Wastewater treatment technologies for the removal of emerging contaminants are membrane bioreactors, ozonation and photocatalytic processes, constructed wetlands, advanced sorbents and nanotechnology, artificial recharge (Barceló et al., 2008; Radjenovic et al., 2009). None of these processes though is entirely successful in the complete removal of contaminants. Ozone treatment, for example, typically transforms chemical compounds but does not mineralize them entirely (Stalter et al., 2009).

1.2 Fate of contaminants in the agro-ecosystem

Application of municipal biosolids on agricultural lands, as a source of crop nutrients and organic matter, is a common farming practice in many countries and jurisdictions (European Commission, 2001; Mantovi et al., 2005; Edwards et al., 2009). Furthermore, municipal wastewater reuse, through irrigation of agricultural land with reclaimed water, is an important supplement to water scarcity

worldwide, especially in arid regions. During the dry season, streams rely almost entirely on STPs effluents for flow, and effluents are extensively used in irrigation and even for recharging drinking water aquifers. However fields amended with biosolids and irrigated with reclaimed water risk to affect water quality by runoff of contaminants of emerging interest (Lee et al., 2007; Topp et al., 2008). The most worrying consequence is that these contaminants are continuously exposed to humans, with different possible pathways: ingestion of food plants cultivated on land irrigated with reclaimed water, ingestion of meat/animal products from animal pasture on contaminated land, ingestion of drinking water produced from groundwater polluted by reclaimed water, inhalation of volatile contaminants during irrigation processes (Figure 2).

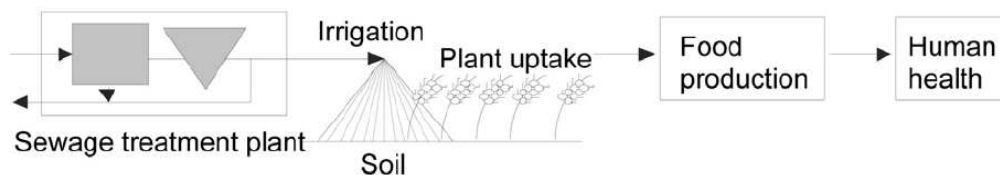


Figure 2 - Exposure pathway of chemicals to humans via agricultural irrigation (Weber et al., 2006).

It has been reported that the use of reclaimed water for soil irrigation can result in the presence of pharmaceuticals in soil, in concentrations that vary through the irrigation season. Some compounds persist for months after irrigation, and accumulate in soil. It was demonstrated that soil samples collected before the irrigation season contained pharmaceuticals, presumably left over from the previous year's irrigation, including erythromycin, carbamazepine, fluoxetine (an antidepressant), and diphenhydramine (a common antihistamine). Several of the pharmaceuticals detected increased in concentration during the study, suggesting that the soil retained or adsorbed the pharmaceuticals (Kinney et al., 2006).

Agricultural land receives also other types of organic waste, such as solid and liquid manure from intensive livestock farming sites, and effluents from intensive aquaculture systems, in order to recycle nutrients and water for crop production. However hormones, antibiotics and veterinary medicines are used extensively in livestock production and, after application to animals, the drug may be adsorbed and partially metabolized before being excreted with urine and faeces. Once the

resulting manure or slurry is applied to land, the medicines and their metabolites may run off into surface waters or leach to groundwater, where they may impact the environment as well as human health (Koschorreck et al., 2002). The re-use of manure is therefore a significant source of emerging contaminants in agricultural land (Kolpin et al., 2002; Christian et al., 2003; Kumar et al., 2004). Aquaculture systems, where the use of veterinary drugs is ordinary and necessary, also contribute to the dispersion of pharmaceuticals into soil (Kupka-Hansen et al., 1992).

1.3 Why are emerging contaminants a concern?

For most emerging contaminants there is currently little information regarding their potential toxicological significance in ecosystems. The very low concentrations in the environment, far below the doses employed for medical treatments, avoid the detection of any biological effects with acute toxicity tests (Boxall et al., 2003). The effects of these contaminants are especially related to long-term and low-level environmental exposures, and they do not need to be persistent to cause negative effects, due to the continuous introduction of assumed drugs into the environment. The chronic nature of exposure to trace concentrations of pharmaceuticals, the synergistic effects of mixtures of unrelated chemicals (Cleuvers, 2003), and to what extent drugs can be transferred to humans through food-chain biomagnification, are mostly unknown and advise caution.

The reason why pharmaceuticals are problematic as environmental micropollutants, is that they are developed with the intention of performing a biological effect. Certain pharmaceuticals are designed to modulate endocrine and immune systems and cellular signal transduction and as such have obvious potential as endocrine disruptors in the environment. Antibiotics are meant to produce direct effects on bacteria, and consequently have the potential to alter the microbial community structure, change the growth, enzyme activity and diversity of microbes (Schiermeier, 2003), and select for those few resistant bacteria in any given population, which then reproduce and create an increasingly resistant population through successive generations (Castiglioni et al., 2008; Farrell, 2009). The use of biological systems for the treatment of antibiotic production wastewater creates an ecosystem that contains much higher concentrations of

antibiotics than normal aquatic environments, and thus may be an important reservoir of antibiotic-resistant bacteria. In a survey of a wastewater treatment plant that received effluent from a penicillin G production facility, Li et al. (2009) demonstrated that, compared with upstream samples, effluent and downstream samples showed significantly high levels of resistance for almost all the tested antibiotics.

The effects of pharmaceuticals on water bacterial communities are principally a reduction from 50 to 70% of the bacterial number in water and sediments and therefore inhibition of bacteria responsible of anaerobic degradation of organic matter: reduction of nitrification processes and of sulphate-reducing activity. Concentrations of 12.5-75 mg L⁻¹ of oxytetracycline, a broad spectrum antibiotic, have been found to be inhibitive of nitrification, and lead to a build-up of toxic ammonia and nitrite (Klaver et al., 1994). Oxytetracycline and flumequine inactivate completely sulphate-reducing bacteria after 7 days of medication (Kupka-Hansen et al., 1992; Smith et al., 1994).

Exposure to waterborne pollutants may cause health risks, such as contamination of aquatic food sources and of agricultural products (Weber et al., 2006). Hence, any measures taken to prevent the chemical pollution of surface and groundwater resources will not only improve ecosystem health, but will also benefit both the production of clean water and safe food for human consumption (Schwarzenbach et al., 2006). Understanding the sources, transport, and fate of emerging contaminants is therefore essential to provide information to eventually expand the range of pollutants that should be monitored in effluent discharges and the implementation of the guidelines.

Environment contamination with new pollutants may result in changes in the microbial ecology, possibly changing the types of bacteria that carry out important ecosystem processes such as nutrient transformations and biomass decomposition. Microbial biodiversity has in fact a functional importance in the maintenance of soil and water biological processes, because most of the transformations involved in biogeochemical cycles are mediated exclusively by microorganisms. It has been reported that shifts in microbial community structure, associated with a reduction in microbial biodiversity, lead to losses of functional stability (Griffiths et al., 2004; Girvan et al., 2005).

Nitrogen is an essential element for crop growth and a key agricultural input. The fixation of N_2 from the atmosphere, in which it is reduced to ammonia in an energy-demanding process, is due principally to microbial activity. The oxidation of ammonia to nitrate via nitrite by autotrophic nitrifying bacteria is a key process in agricultural/natural ecosystems and wastewater treatment (Jordan et al., 2005). The first step, the oxidation of ammonia to hydroxylamine, is catalyzed by aerobic chemoautotrophic ammonia oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB). Activity and dynamics of the nitrifying bacterial population have been largely used in environmental studies (Hermansson and Lindgren, 2001; Limpiyakorn et al., 2005; Molina et al., 2007). Studies have shown that AOB and NOB are less competitive than the heterotrophic bacteria for oxygen and growing space and are sensitive to environmental inhibition (Van Benthum et al., 1997; Juliastuti et al., 2003; Limpiyakorn et al., 2005; Pagga et al., 2006). Ammonium oxidation by autotrophic ammonia-oxidizing bacteria (AOB) is a key process in agricultural ecosystems and wastewater treatment. Denitrification occurs in many distantly related species of microorganisms (Zumft, 1992), thus also bacteria with this physiological capability may be used as functional markers for ecological studies (Gregory et al., 2003). Bacterial communities constitute the basis of food webs and are responsible for organic matter transformations and mineral recycling. Ecosystem functions that depend on microbial activities can suffer from chemical exposures if microorganisms are sensitive to the toxic effects of pollutants (Ogunseitan, 2000). Given the various sensitivities of different microorganisms to toxic chemicals, there is a growing interest in microbial toxicity testing at the community or ecosystem level, and in including bacterial community responses in the environmental risk assessment of toxic pollutants (Brandt et al., 2004).

1.4 Microbial ecology approach

Traditional parameters used for the Environmental Risk Assessment (ERA) of pharmaceuticals are chemodynamic and physico-chemical properties such as solubility, K_{ow} (octanol/water partition constant), K_d (soil-water partition constant), K_{oc} (soil organic carbon/water partition constant) and DT_{50} (degradation half-life) in soil and water, PEC (predicted environmental concentration), PNEC (predicted no-effect concentration). Furthermore ecotoxicological effects of

pharmaceuticals on non-target organisms in water and soil are assessed with standard acute and chronic ecotoxicologic tests on freshwater and marine organisms (e.g. *Daphnia magna*, rainbow trout) and soil organisms (e.g. *Eisenia fetida*, *Enchytraeus crypticus*, *Caenorhabditis elegans*, *Folsomia candida*).

Pollution may influence soil and water quality and productivity but little is known on the effects on microbial communities, and consequent impacts on functioning. Due to their small size, large numbers, and ubiquitous distribution in the environment, microorganisms are valuable indicators of the occurrence of disturbances due to exogenous physico-chemical stressors. The study of bacterial abundance, vitality and community structure are among the most useful tools developed in microbial ecology for direct characterization of target populations, in their natural environment, avoiding cultivation. The assessment of variations in microbial community structure is of basic importance to permit to evaluate the impact of an environmental stressor. At the organism level, the presence of a certain indicator bacteria can indicate sources of pollution into an environment, but the molecular-level responses of autochthonous microorganisms to changes in ambient conditions are more critical for ecosystem health assessment. There is a wide array of molecules, including nucleic acids, lipids, and proteins, that is useful for diagnosing microbial responses to pollution and for monitoring environmental management strategies. The presence of toxic chemicals in microbial ecosystems, for example, induces the synthesis of detoxifying or degradative enzymes and certain stress proteins (Figure 3). Effects due to chemical toxicity tend to narrow the spectrum of microbial diversity because organisms that are not capable of resisting the toxic effects either die or enter a static metabolic phase, leaving those that have evolved resistance mechanisms, that are capable of utilizing the excess chemicals as nutrients, to proliferate and become dominant members of the impacted ecosystem (Ogunseitan, 2000). Protein molecules mediate these effects by virtue of the ability of each species to synthesize degradative enzymes or otherwise engage in repair mechanisms through the activities of stress proteins and modified structural components (Ogunseitan, 2000). Monitoring these proteins provides information on toxic chemical fates (biodegradative enzymes) and effects (toxicity-induced changes in protein profiles). Complex microbial communities may therefore serve as ideal and ecologically relevant toxicity indicators (Brandt et al., 2004).

A number of microbiologically driven processes has been proposed to evaluate the effects of xenobiotics on ecosystems (Wagner-Döbler et al., 1992; Nazaret et al., 1994; Moyer et al., 1994; Griebler and Slezak, 2001). Proteins, genes, metabolites, or lipids that, when expressed, present a pattern of molecular change in an organism in response to a specific environmental stressor, can be defined as environmental biomarkers.

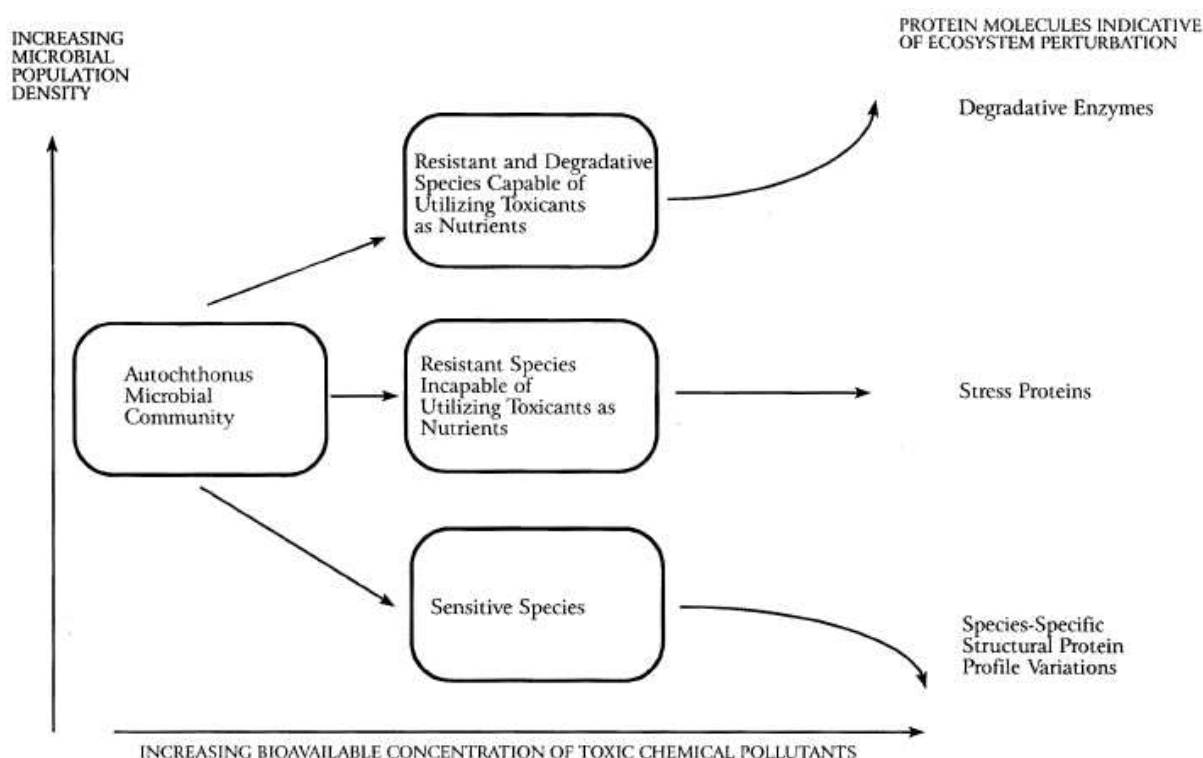


Figure 3 – Schematic representation of microbial community analysis in response to environmental perturbations. Toxic chemicals, for example, can cause changes in microbial population densities and diversity (Ogunseitan, 2000).

The evaluation of bacterial biodiversity is mainly limited by their small size, by the absence of distinguishing phenotypic characters, and by the fact that most of these organisms cannot be cultivated (Torsvik et al., 2002). The number of techniques to study microbial communities has increased exponentially over the last 20 years and the advent of culture-independent methods, such as molecular biological techniques, has changed the view of microbial diversity (Rossello-Mora and Amann, 2001). Among these techniques it is possible to distinguish between those which are primarily based on the use of Polymerase Chain Reaction (PCR),

and those that are non-PCR-based (Figure 4). PCR uses specific primers to amplify a DNA target sequence. The bacterial 16S rDNA gene is today the most commonly used for assessing overall diversity in microbial communities and for studying the phylogeny of microorganisms. Sequence variations in the PCR fragments are detected either by a cloning/sequencing analysis, which provides a complete characterization of the fragments, or by an electrophoretic analysis, which provides a visual separation of the mixture of fragments. Fragments separation is based on sequence polymorphism, in Denaturing Gradient Gel Electrophoresis (DGGE) or length polymorphism, in Terminal-Restriction Fragment Length Polymorphism (T-RFLP) and Automated Ribosomal Intergenic Spacer Analysis (ARISA). DGGE is frequently used in environmental studies (Ibekwe et al., 2001; Guo et al., 2009). Quantitative Polymerase Chain Reaction (qPCR and qRT-PCR) has become a commonly used technique for the detection and quantification of microorganisms in the environment for its high sensitivity at low concentrations (Dionisi et al., 2003; Devers et al., 2004; Zhang and Fang, 2006; Kim et al., 2007). It can be used to detect changes in gene expression patterns induced by adverse conditions, also not requiring prior knowledge of expected contaminants, using non-specific stress responses as general indicators of deleterious conditions (Van Dyk et al., 1995).

Non-PCR-based methods commonly used in environmental studies are epifluorescence microscopy techniques, such as direct count of bacterial abundance (DAPI count) and vitality (Live/Dead cell viability assay), and Fluorescence *In Situ* Hybridization (FISH). FISH uses rRNA-targeted fluorescent probes to investigate the overall taxonomic composition of bacterial communities. Probes can be designed to be complementary to species-, group-, or kingdom-specific target sites.

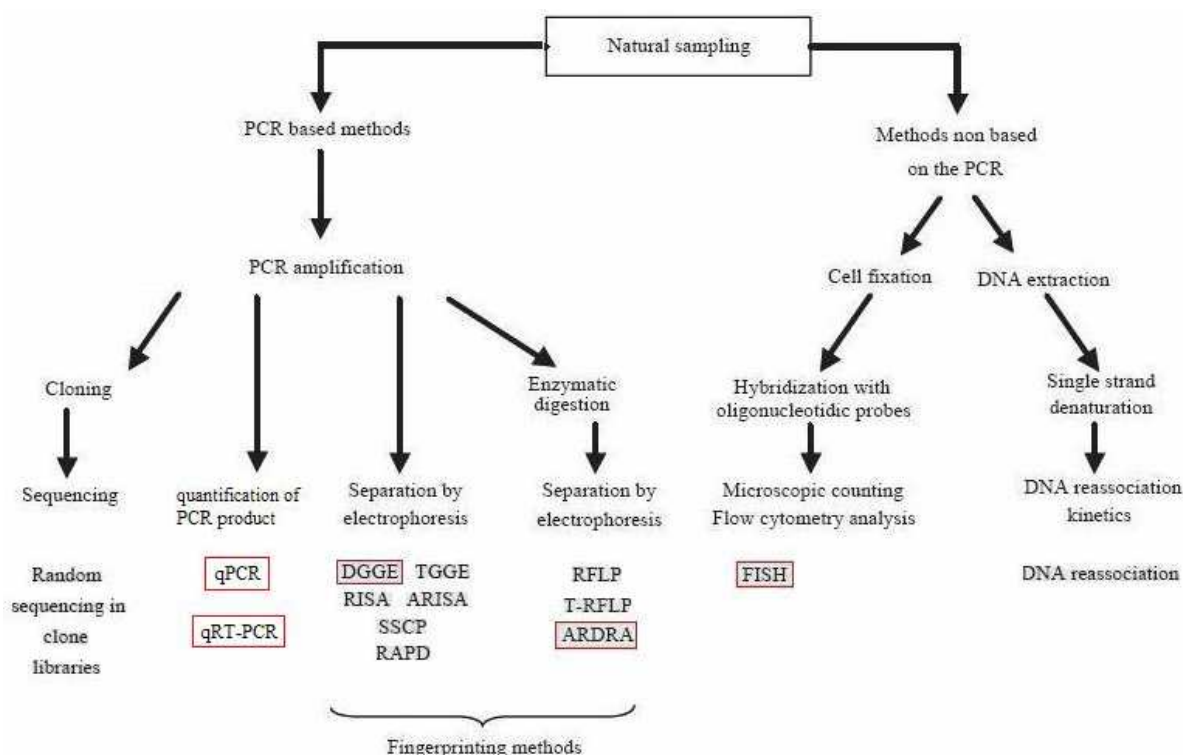


Figure 4 – Diagram of the different molecular approaches for assessing the genetic diversity of microbial communities. qPCR and qRT-PCR (quantitative PCR and quantitative reverse transcriptase-PCR), DGGE (Denaturing Gradient Gel Electrophoresis), ARDRA (Amplified Ribosomal DNA Restriction Analysis) and FISH (Fluorescence *In Situ* Hybridization) were used in this study (modified from Dorigo et al., 2005).

Each of the above mentioned techniques can provide different information for the analysis of environmentally significant genes in microbial communities exposed to toxic chemicals and direct detection of genes involved in maintaining key biochemical functions at the microbial level (Ogunseitan, 2000).

1.5 Legislation in the European Union

During the past decades, the impact of chemical pollution has focused almost exclusively on conventional ‘priority pollutants’, especially those acutely toxic/carcinogenic pesticides and industrial intermediates displaying persistence in the environment. However Daughton and Ternes (1999) have reported that the amount of pharmaceuticals and personal care products entering the environment annually is about equal to the amount of pesticides used each year. Governments now regulate the use and disposal of toxic chemicals more rigorously than in the past, and several forms of legislation have alternated in the past decades to control water pollution in the European Union. The first steps in the European water

legislation have focused mainly on quality standards for certain types of waters (bathing waters, aquaculture and drinking waters), leading to the stipulation of the Drinking Water Directive and the Bathing Water Directive.

Within the European Union, the quality of water for human consumption is determined by the Drinking Water Directive (Council Directive 98/93/EC). Of the 48 parameters within the directive, none is related to pharmaceuticals.

The nutrients dimension was then added to water protection with the Urban Wastewater Treatment Directive of 1991 (Council Directive 91/271/EEC), concerning urban wastewater treatment.

The Nitrates Directive (91/676/EEC) sets out clear rules for nitrates pollution from agriculture, one of the main sources of groundwater pollution as well as of eutrophication of surface waters in many regions of Europe.

In 2000, the Water Framework Directive (2000/60/EC) has expanded EU water policy to all waters and addresses all sources of impacts. It defines the ecological quality according to hydro morphological, physico-chemical and biological (biodiversity to the three levels: genetic, of population, of community) parameters, and priority pollutants concentrations in water, sediments and organisms. The Directive on Priority Substances (2008/105/EC) identifies 33 substances or groups of substances, which have been shown to be of major concern for European Waters, for the adoption of control measures over the next 20 years (http://ec.europa.eu/environment/water/water-framework/priority_substances.htm). Further 14 substances were identified as being subject to review for identification as possible priority hazardous substances. The list includes selected chemicals, plant protection products, biocides, metals and other groups like Polyaromatic Hydrocarbons (PAH) that are mainly incineration by-products and Polybrominated Biphenylethers (PBDE) that are used as flame retardants. Additionally member countries have undertaken their own national reviews to identify emerging future contaminants. The much wider range of emerging pollutants that are now widely used is not included in the list, however the priority substance list will be updated every 4 years and has identified future emerging priority candidates.

Since 2007, regulation on chemicals and their safe use is established by the European Community (EC 1907/2006) with the REACH legislation (Registration, Evaluation, Authorization of Chemicals). REACH regulates the large number of

substances that have entered the market in Europe in the last years, sometimes in very high amounts, for yet there is insufficient information on the hazards that they pose to human health and the environment.

Furthermore, European guidelines for use of reclaimed water are generally limited to defining risks associated with microbial organisms, bulk parameters such as chemical oxygen demand (COD), biochemical oxygen demand (BOD), pH and total suspended solids (TSS). These parameters exclude the monitoring of specific chemical concentrations.

The European Agency for the Evaluation of Medicinal Products (EMA) coordinates the existing scientific resources of the Member States of the EU in order to evaluate and supervise medicinal products for both human and veterinary use throughout the entire EU.

1.6 Bioremediation

Molecular ecological information is especially useful for the development of strategies to improve bioremediation, in which the metabolic potential of microorganisms is used to clean up contaminated environments (Watanabe, 2001). In the last years molecular tools have facilitated the study of natural microbial populations without cultivation, including the fraction of microorganisms that have the ability to degrade certain xenobiotics.

Bioremediation employs living organisms, most often microorganisms, plants, or both to degrade, detoxify, or sequester toxic chemicals from natural waters and soils. It can be used to treat soil, sediment, sludge, water, or even air. Treatments can be either *ex situ*, involving the removal of contaminated materials from a polluted site prior to treatment, or *in situ*, if contaminants are treated without moving them to a treatment facility. Bioremediation treatments include: bioaugmentation, by augmenting natural systems with exogenous biological materials, usually natural microorganisms or plants grown to large numbers in fermenters or greenhouses; biostimulation, the use of nutrients, substrates or environmental conditions to stimulate the naturally occurring organisms that can perform bioremediation; bioreactors, treatment of a contaminated substance in a large tank containing organisms or enzymes; bioventing, involves the venting of oxygen through soil to stimulate the growth of natural and introduced bioremediation organisms; composting, involves mixing contaminated materials

with compost containing bioremediation organisms; land farming, the use of farming tilling and soil amendment techniques to encourage the growth of bioremediation organisms in a contaminated area. Finally, abiotic processes sometimes can be used in combination with biotic processes to degrade particularly recalcitrant molecules. Examples of abiotic catalysts that may enhance biodegradative processes include ultraviolet light, inorganic reductants, and Fenton reagent (iron and hydrogen peroxide). The bioremediation industry has developed many novel approaches for monitoring and quantifying bioremediation processes (Crawford, 2006) to offer an efficient, cheap and biocompatible option for decontamination of polluted ecosystems.

The biodegradative environmental fate of contaminants can be determined through the integration of field, laboratory, and modelling efforts (Hooper et al., 2002). The National Research Council (1993) has recommended three criteria for demonstrating intrinsic remediation: documenting a decrease in contaminant concentrations at the site, showing experimentally that microorganisms in site samples have the potential to transform the contaminants under expected site conditions, developing evidence showing that the biodegradation potential is actually realized in the field.

Among bacteria, the degradation of recalcitrant pollutants is of great environmental significance. A wide variety of bacteria able to utilize xenobiotics as a source of energy and capable of degrading a broad range of pollutants has been isolated (Gu and Berry, 1992; Topp et al., 2000; Gu and Mitchell, 2006; Singh and Walker, 2006; Yoon et al., 2006; Miyauchi et al., 2008), and many have been exploited in pollutant biodegradation and wastewater treatment (Bryers, 1994; Osswald et al., 1995; Sharp et al., 1998). *Pseudomonas* sp. ADP is one of the best studied s-triazine-degrading bacteria (Mandelbaum et al., 1995; Martinez et al., 2001; Moràn et al., 2006), *Arthrobacter aurescens* TC1 is able to degrade a variety of pollutants, among which the herbicide glyphosate, mixed bacterial cultures in a consortium can show degradation ability of various pollutants, even though their individual components can be unable to utilize the chemical as energy source (Mandelbaum et al., 1993; De Souza et al., 1993).

Furthermore, among the genus Basidiomycetes, the so called white rot fungi (WRF) are capable of degrading a lignocellulose substrate by producing three types of extracellular enzymes, often referred to as Lignin Modifying Enzymes

(LMEs), and they are Lignin Peroxidase (LiP), Manganese-Dependent Peroxidase (MnP) and Laccase (Lac). LiP oxidises methoxyl groups on aromatic rings, MnP and Lac are able to oxidise phenolic substrates. As the enzymes are non-specific, they have been found capable of degrading a wide variety of chemical compounds like DDT, PCB, lindane, dioxin, benzopyrene, cyanides, azides, CCl₄ and pentachlorophenol (Singh et al., 1999; Lu et al., 2009). The main fungus studied is *Phanerochaete chrysosporium*, and also studied extensively are *Trametes versicolor*, *Pleurotus ostreatus*, *Phanerochaete sordida*, *Trametes hirsutus*, and *Fusarium culmorum*.

2. AIM OF THE THESIS AND SELECTED PHARMACEUTICALS

The aim of the present thesis is to assess the impact of selected emerging contaminants on the microbial community of different water and soil ecosystems, selected for the study. This work is structured in three parts, each regarding the fate of different pharmaceuticals.

Chapter 1 includes the main part of the work, it regards the fate and removal of the antiviral Tamiflu (oseltamivir carboxylate, OC), recommended for the treatment of cases of avian and swine influenza. Tamiflu is predicted to reach the water system because resistant to biodegradation in wastewater treatment plants. Contrasting environmental samples were chosen for laboratory experiments: three surface water ecosystems of Italy, such as an irrigation canal Canale Emiliano Romagnolo (**paragraph 1**), River Po and Venice Lagoon (**paragraph 2**); two surface water ecosystems of Japan, River Furukawa and Lake Biwa (**paragraph 3**); and samples of activated-sludge-mixed liquor from the municipal wastewater treatment plant of the city of Bologna and the effluent water of the plant (**paragraph 4**). Besides degradation and mineralization of OC during incubation time, the effect of OC on the bacterial community structure was determined by fingerprinting techniques (ARDRA and DGGE), qPCR, qRT-PCR, and epifluorescence microscopy techniques (FISH, bacterial abundance and vitality). Furthermore, bacterial strains growing on oseltamivir as sole carbon source were isolated and tested for degradation capacities. A bioremediation strategy was performed to evaluate the capability of a white rot fungus, *P. chrysosporium*, to degrade the antiviral.

In **Chapter 2** the destiny of common use human and veterinary antibiotics, such as ciprofloxacin, erythromycin, sulfamethoxazole, was monitored in the aforementioned wastewater treatment plant and effluent. Bioremediation with *P. chrysosporium* was tested for degradation processes acceleration.

Chapter 3 concerns the fate of two veterinary pharmaceuticals, doramectin and oxbendazole, common parasitic treatments in farms, in contaminated soils, and the effect on the bacterial community structure.

The work for this thesis was conducted principally in the University of Bologna (Department of Agroenvironmental Sciences and Technologies), with the contribution of national and international collaborations. A part of the research regarding the antiviral Tamiflu (Chapter 1) was conducted with the Water Research Institute (IRSA, CNR of Rome), in particular epifluorescence microscopy techniques. Collaboration with the Department of Chemistry of Umeå University (Dr. Jerker Fick) regarded chemical analysis conducted in Chapter 1 and 2. The work in Chapter 3 was conducted during a 9 month period (January 2007 - April 2007; September 2008 - March 2009) spent in Complutense University of Madrid, in the Faculty of Veterinary, under the supervision of Prof. Margarita Martín.

2.1 Fate and removal of the antiviral drug oseltamivir (Tamiflu) in superficial water

During the past years influenza A viral infections have posed serious risks to public health. Since 2003, 286 cases of ‘avian’ influenza H5N1 human deaths have been confirmed by the WHO, and worldwide more than 213 countries have reported laboratory confirmed cases of ‘swine’ influenza H1N1, including at least 16226 deaths. The available options to control influenza A viruses are limited. Health agencies all over the world have been forced to adopt strategies for containing the viruses and to protect the health of the public. Although vaccination is the primary strategy for prevention, neuraminidase inhibitors are the drugs of choice for the treatment and the prevention of influenza A illness in children and adults. The World Health Organization (WHO, 2006) has recommended the use of the antiviral drug Tamiflu (oseltamivir phosphate),

produced and marketed by F. Hoffman-La Roche Ltd (Basel, Switzerland), for the treatment and post-exposure prophylaxis in a pandemic scenario. Hundreds of million of course of Tamiflu have been stockpiled worldwide since 2003, and in the last year sales of the antiviral have further exploded. Oseltamivir phosphate (OP) [ethyl-(3*R*,4*R*,5*S*)-4-acetamido-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylate] is the prodrug of the active metabolite oseltamivir carboxylate (OC) [(3*R*,4*R*,5*S*)-4-acetamido-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylic acid], a specific inhibitor of influenza A and B virus neuraminidase (Kim et al., 1997; Li et al., 1998). OC reduces viral infection by binding to the highly conserved active site of the neuraminidase of the virus, inhibiting the release of progeny virions from the surface of infected cells (Bardsley-Elliot and Noble 1999). OC has been shown to be clinically active for the treatment and chemoprophylaxis of influenza in adults and in children (Ward et al., 2005). Pharmacological studies have demonstrated that after oral administration of OP and absorption in the gastrointestinal tract, it is converted by the hepatic esterases to OC (Figure 5). More than 80% of each oral dose of OP is eliminated by renal excretion as OC (Ward et al., 2005).

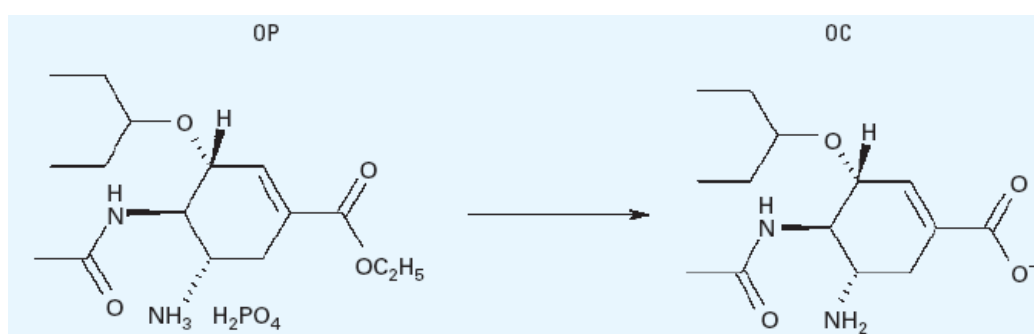


Figure 5 – Structure of the prodrug oseltamivir phosphate (OP) and the active form oseltamivir carboxylate (OC) (Singer et al., 2007).

The OC molecule has amine and carboxylate groups that impart hydrophilicity, a low partition coefficient (log P 1.1), and high water solubility (588 mg mL⁻¹ at 25°C) (American Hospital Formulary Service, 2006). These physico-chemical features minimize loss by sorption to sewage sludge during wastewater treatment. A recent study conducted in Sweden by Fick et al. (2007) demonstrated that OC is not completely removed during conventional sewage water treatments which include mechanical, chemical and biological (activated-sludge) processes. Consequently, in case of urban areas with a large number of patients receiving

Tamiflu, the potential risk of OC to contaminate the aquatic ecosystem is expected (Fick et al., 2007; Singer et al., 2007, 2008). Predicted environmental concentration (PEC) of OC, calculated in catchments with particularly low flow and high populations, would be over $20 \mu\text{g L}^{-1}$, which is significantly higher than that observed for most other pharmaceutical contaminants (Singer et al., 2008). This assumption was recently confirmed in two studies conducted in Japan, OC was detected in a conventional activated-sludge-based STP discharge in the concentration of 293.3 ng L^{-1} , and in the receiving river water samples in the concentration of $6.6\text{--}190.2 \text{ ng L}^{-1}$ during the peak of the flu season (Ghosh et al., 2010). Söderstrom et al., (2009) detected no OC in Japanese surface water systems before the flu-season, but $2\text{--}58 \text{ ng L}^{-1}$ were found in the samples taken during the flu season.

Chronic ecotoxicity tests, conducted in light of the 2006 EMEA guidelines on environmental risk assessment for human pharmaceuticals, revealed that the level of concern regarding ecotoxicity is quite low. The preliminary no observed effects concentrations (NOECs) resulted in a PNEC of $100 \mu\text{g L}^{-1}$, applying an assessment factor of 10 (Singer et al., 2007). Considerable concerns are the potential inhibition of non-target neuraminidases in different organisms than influenza viruses, and the fact that the presence of OC into rivers can be a risk for the generation of OC resistance in influenza viruses. Recent analysis of isolated A viruses revealed a high increase in Tamiflu-resistant strains, in different countries worldwide (Hurt et al., 2009).

2.2 Fate and removal of common use antibiotics in WWTP

Antibiotics are used extensively in human and veterinary medicine, as well as in aquaculture, for preventing or treating microbial infections. The risk to contaminate soil or surface water run-off after application of manure, or in the use of sewage sludge for land amendment, is high, increasing the selective pressure for resistant bacteria (Kümmerer et al., 2009b). Common use antibiotics of emerging concern, most likely to cause environmental problems (Zuccato et al., 2006), have been used in this work, for a degradation and bioremediation study (Figure 6).

Ciprofloxacin is a broad-spectrum fluoroquinolone antibacterial drug, commonly used for the treatment of bacterial infections, in particular of the urinary and

respiratory tracts. Erythromycin is a macrolide antibiotic, used to treat several types of infections (respiratory tract infections, skin infections, acute pelvic inflammatory disease, erythrasma, etc.). Sulfamethoxazole is a sulfonamide bacteriostatic antibiotic, often used in combination with trimethoprim. Excretion rates and further details on properties and metabolism of antibiotics can be found on <http://pubchem.ncbi.nlm.nih.gov/>.

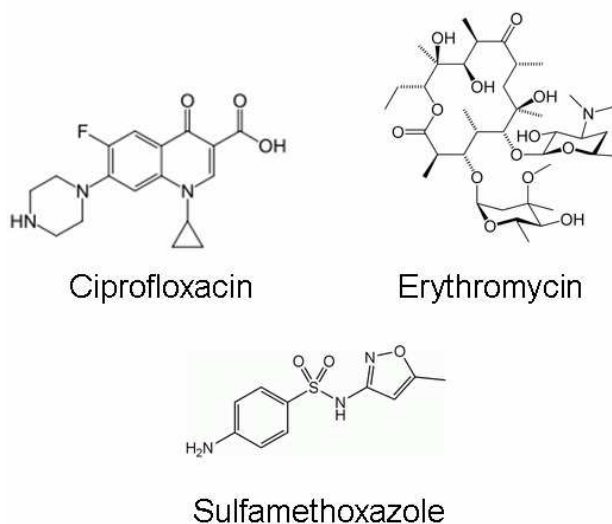


Figure 6 - Human and veterinary common use antibiotics used in the study.

2.3 Fate and removal of veterinary pharmaceuticals in soil

Two veterinary antimicrobials were selected in this study to assess their effects on a contaminated soil bacterial community: doramectin and oxibendazole (Figure 7).

Doramectin is a macrocyclic lactone, potent anthelmintic, for the treatment of parasites such as gastrointestinal roundworms, lungworms, eyeworms, grubs, sucking lice and mange mites in cattle. It is an endectocide molecule, for the activity against ecto- and endo-parasites (Shoop et al., 1995). It has low mammalian toxicity and formulations are convenient to use, hence it is extensively used worldwide in veterinary medicine. However the occurrence and persistence of residues of the drug brings the need for continued monitoring of its fate. Residues of doramectin ($79.8 \mu\text{g kg}^{-1}$) have been found in sheep milk at 3 days post-treatment, and were still detectable for 30 days after treatment (Danaher

et al., 2006). In faeces of livestock, residues may represent a potential risk for soil microfauna (Kolar et al., 2008).

Oxibendazole is a benzimidazole, broad spectrum anthelmintic, used in veterinary medicine to protect porcine species from roundworms, strongyles, threadworms, pinworms and lungworm infestations. After administration of the drugs, large amounts of unchanged product are excreted by urine and faeces, particularly during the first weeks after treatment (Lifschitz et al., 2000), and can reach agricultural ecosystems through the application of manure on soil.

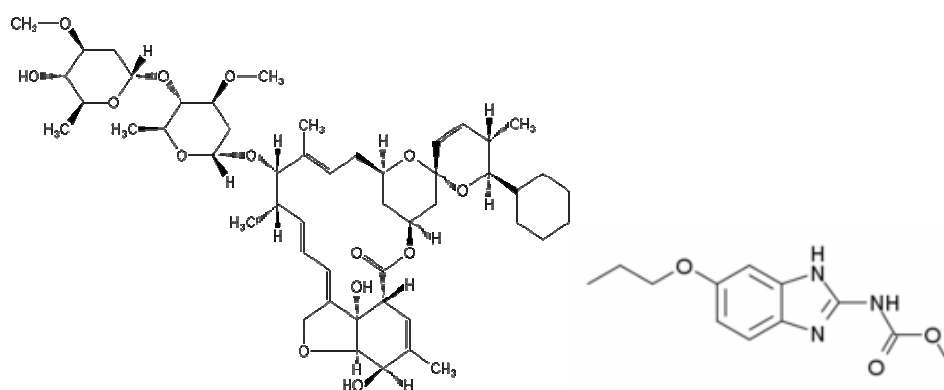


Figure 7 – Veterinary pharmaceuticals doramectin (left) and oxibendazole (right).

3. MATERIALS AND METHODS

3.1 CHAPTER 1 – The antiviral drug Tamiflu

3.1.1 Chemical analysis

a. Degradation analysis by HPLC

Oseltamivir carboxylate (OC) concentration in incubated water samples of the CER irrigation canal was determined by HPLC after derivatization with 20 mM naphthalene-2,3-dialdehyde (Sigma-Aldrich Italia s.r.l., Milan, Italy) and 20 mM potassium cyanide (Ultra Scientific Italia s.r.l., Bologna, Italy) as described in Eisenberg and Cundy (1998). Aliquots of the derivatized mixtures were analysed by a chromatography system equipped with a 250 x 0.46 mm Prodigy ODS-2 column (Phenomenex Inc., Torrance, CA), and an RF-10AXL spectrofluorometric detector (Shimadzu Italia s.r.l., Milan, Italy). Isocratic elution was carried out at 40 °C, and the eluent flow was set at 1.0 mL min⁻¹ with 50 mM sodium acetate in acetonitrile/water (27 : 73, v/v). Detection of OC was achieved by setting the detector at excitation and emission wavelengths of 420 and 472 nm, respectively. OC was quantified on the basis of external standards. OC was obtained from analytical grade OP (≥ 99% purity; Sequoia Research Product, Pangbourne, UK) by chemical hydrolysis at elevated pH. Samples from flasks containing water and sediments were extracted with ethanol, centrifuged at 5000 g for 10 min, redissolved in 50 mM monosodium phosphate and analysed as described above. Recoveries of OC from water and water/sediment samples were 97.1 and 87.7%, respectively.

b. Degradation analysis by LC-ESI-MS/MS

OC degradation in the further experiments was assessed following the procedure described in Fick et al. (2007), samples were extracted by solid phase extraction and analyzed by liquid chromatography/electro spray tandem mass spectrometry. Briefly, samples were acidified to pH 3, filtered through a 0.45-μm filter and loaded on a Strata-X-C (200 mg, 6 mL) column (Phenomenex Inc. Torrance, CA, USA). Eluate was concentrated and reconstituted in acetonitrile/H₂O (1:1) containing 0.1% formic acid. Aliquots (10 μL) were injected into a LC-ESI-

MS/MS equipped with C₁₈ column (YMC Inc. Wilmington, NC), a P40000 HPLC pump (Thermo Scientific Inc., Waltham, MA, USA) and a LCQ Duo ion-trap mass spectrometer (Thermo Scientific Inc.). Oseltamivir carboxylate (OC) was obtained from F. Hoffmann-La Roche Ltd (Basel, Switzerland).

c. Mineralization analysis

Mineralization of [U-ring-¹⁴C]-OC was measured in biometers, which consisted in 250-mL flasks equipped with suspended glass vials containing 4 mL of a 1 M NaOH solution to trap ¹⁴CO₂. Aliquots of water (50 mL) were transferred into each flask under aseptic conditions and spiked with a solution of unlabeled (chemical purity > 98%) and ¹⁴C-OC (radiopurity > 97.9%, specific activity 4.96 MBq mg⁻¹) to give a final concentration of 20 µg L⁻¹ (1.5 kBq mL⁻¹). Radiolabeled OC was provided by F. Hoffmann-La Roche Ltd (Basel, Switzerland). Water samples containing 5 and 10% (w/v) sediments were included in the study. Samples consisting in autoclaved water and water/sediments were used as controls. Samples were incubated for 21 days at 20 °C on an orbital shaker (125 rpm) in the dark. Trapped ¹⁴CO₂ was determined by mixing a 1-mL aliquot of NaOH solution with 4 mL of Hi Safe 3 scintillation cocktail (PerkinElmer, Boston, MA, USA), and the amount of radioactivity was measured by liquid scintillation counting (LSC) using a Wallac 1490 liquid scintillation counter (Wallac Oy, Turku, Finland). Prior to analysis, samples were kept in the dark for 12 hours.

The experiment was conducted in triplicate. Data of the degradation and mineralization study were analyzed by analysis of variance. Means were separated by Fisher's least significant difference (LSD) and significant differences were detected at the P = 0.05 level.

d. Bioavailability and sorption isotherms

At the end of the incubation period, samples of the mineralization study were transferred into 50-mL centrifuge tubes, shaken for 1 hour and centrifuged at 5000 g for 10 min. The total volume of supernatant was measured and total radioactivity determined by LSC, mixing triplicate 1-mL aliquots with 4 mL of HiSafe 3 Scintillation Cocktail. Pellets were sequentially extracted with 0.1 M CaCl₂, and acetonitrile. For each extraction, pellets were dispersed by vortexing,

shaken for 3 hours and centrifuged at 5000 g for 10 min. Total radioactivity in the supernatants was determined by LSC. Finally, remaining ^{14}C -residues were determined by combusting triplicate subsamples of ACN-extracted pellet using a Packard 306 (Packard. Instrument Co., Sterling, VA, USA). Sorption isotherms of OC on sediments of the River Po and Venice Lagoon were determined by the batch equilibrium method. Aliquots (2 g, air-dried basis) of each sediment were weighed into 50-mL glass centrifuge tubes and a 10-mL aliquot of ^{14}C -OC solution, prepared in 0.01 M CaCl_2 , was added. Sorption isotherms were determined using triplicate samples at five initial OC concentrations, ranging from 20 to 100 $\mu\text{g mL}^{-1}$. Radiolabeled OC was added to unlabeled solutions to give an initial radioactivity of approximately $3 \times 10^{-3} \mu\text{Ci mL}^{-1}$. Tubes were sealed with teflon-lined caps, mechanically shaken at 20 °C for 14 hours, and samples were centrifuged at 5000 g for 10 min. Aliquots (5 mL) of supernatant were removed, filtered through a 0.2- μm filter, and radioactivity in 1-mL fractions was determined by LSC. Preliminary investigations showed that equilibrium was attained in less than 14 hours and that there was no significant OC sorption to centrifuge tubes. The amount of sorbed OC was calculated from the concentration differences between the supernatant of the equilibrated solutions and those of the corresponding initial solutions. Sorption data were fitted to the log form of the Freundlich equation:

$$\log C_s = \log K_f + (1/n) \log C_e$$

where C_s is the concentration of OC sorbed ($\mu\text{g g}^{-1}$ sediment), C_e is the equilibrium concentration ($\mu\text{g mL}^{-1}$ solution) and K_f and $1/n$ are the empirical Freundlich constants. Values of K_f and $1/n$ were estimated by linear regression after a log–log transformation.

3.1.2 Microbial analysis

In this work, microbial community level toxicity was tested using a polyphasic approach, involving a range of molecular-based methods, targeting both structure and function of the tested microbial communities.

Potential effects of OC on basic microbiological aspects of the indigenous microbial community of water and sediments were investigated using DNA-based

approaches. All the microbiological analyses were conducted using aliquots taken from samples of the OC degradation study.

Bacterial community structure

e. Bacterial abundance by direct count (DAPI)

The size of the bacterial population in water samples was estimated by direct count, using a fluorescent dye. Total bacterial abundance was calculated by fixing, at different time intervals, aliquots (1 mL) of water with the same amount of phosphate-buffer saline (PBS) containing formaldehyde (2% w/v), Tween 20 (0.5 v/v) and sodium pyrophosphate (0.1 M). In order to separate water bacterial aggregates, a gentle sonication (10 sec, 15 W using a Microson XL2000 ultrasonic liquid processor) was performed on each sub-sample. Samples were then treated with the DNA-binding fluorescent stain 4'-6-diamino-2-phenylindole (DAPI) (1 $\mu\text{g mL}^{-1}$), and filtered onto a 0.22 mm black polycarbonate filter. Cells were enumerated using an epifluorescence microscope (DM LB30, Leica GmbH, Heideberg, Germany), as described in Barra Caracciolo et al. (2005).

f. Bacterial phylogenetic composition by Fluorescence In Situ Hybridization (FISH)

In order to investigate the effects of OC on the bacterial community and to assess if it could be involved in degradation, Florescence *In Situ* Hybridization (FISH) was performed on OC treated and untreated sub-samples collected from degradation microcosms (2 replicates). The phylogenetic composition of the OC-treated and control samples was analyzed at different sampling times (0, 14, 21 and 36 days). For each condition, four sub-samples (1 mL each) were fixed (1:1) with a solution composed of phosphate-buffered saline: 130 mM NaCl; 7 mM Na_2HPO_4 ; 3 mM NaH_2PO_4 ; 2% formaldehyde; 0.5% Tween 20 and 100 mM sodium pyrophosphate. After sonication, samples were filtered on a 0.2 μm polycarbonate membrane. Filters were stored at -20 °C until further processing. FISH of the harvested cells was performed using probes for the identification of the major bacterial phylogenetic divisions found in freshwater (Zwart et al., 2002), such as the *Bacteria* domain, and the phyla of α -*Proteobacteria*, β -*Proteobacteria*, γ -*Proteobacteria*, *Planctomycetes*, *Cytophaga-Flavobacterium*, *Firmicutes*. For this purpose, the Cy3-labelled oligonucleotide probes described in

Table 2 were applied, in accordance with previously published protocols (Barra Caracciolo et al., 2010; Grenni et al., 2009). Further details of the probes are available at <http://www.microbial-ecology.net/probebase> (Loy et al., 2007).

Target Taxa	Probe	Sequence (5'-3')
<i>Archaea</i>	ARCH915	GTGCTCCCCCGCCAATTCCT
<i>Bacteria</i>	EUB338	GCTGCCTCCCGTAGGAGT
<i>Bacteria</i>	EUB338II	GCAGCCACCCGTAGGTGT
<i>Bacteria</i>	EUB338III	GCTGCCACCCGTAGGTGT
<i>α-Proteobacteria</i>	ALF1b	CGTTCGYTCTGAGCCAG
<i>β-Proteobacteria</i>	BET42a	GCCTTCCCACCTTCGTTT
<i>γ-Proteobacteria</i>	GAM42a	GCCTTCCCACATCGTTT
<i>δ-proteobacteria SRB</i> Sulfate-Reducing Bacteria	SRB385	CGGCGTCGCTGCGTCAGG
<i>ϵ-proteobacteria</i>	EPS710	CAGTATCATCCCAGCAGA
<i>Planctomycetes</i>	PLA46	GACTTG CATGCCTAATCC
<i>Planctomycetes</i>	PLA886	GCCTTGCGACCATACTCCC
<i>Cytophaga-Flavobacterium</i>	CF319a	TGGTCCGTGTCTCAGTAC
<i>Actinobacteria</i>	HGC69a	TATAGTTACCACCGCCGT
<i>Firmicutes</i>	LGC354a	TGGAAGATTCCCTACTGC

Table 2 – Cy3-labelled oligonucleotide probes applied in this study for Fluorescence *In Situ* Hybridization analysis. ARCH915, SRB385, EPS710, HGC69a probes were used in the subsequent study described in paragraph 3.3.3. Further details on the above-mentioned probes are available at probeBase (Loy et al., 2007).

The number of cells binding to the probes for each bacterial group was calculated as a percentage of the total DAPI positive cells (500-1000 stained cells). The slides were mounted with a drop of Vectashield Mounting Medium and the preparation examined and counted with a Leica DM 4000B epifluorescence microscope at 1000 x magnification. Experimental data are reported as the

number of cells mL⁻¹, calculated by multiplying the total cell abundance and the percentage of cells detected by each specific probe.

Data were obtained from the mean of four sub-samples. Statistical analysis of the data was done using Kruskal-Wallis One Way Analysis of Variance on Ranks, with significant differences at level of $P < 0.01$.

g. Microbial community structure by Amplified Ribosomal DNA Restriction Analysis (ARDRA)

Samples of the degradation study were used to determine bacterial population changes in response to the presence of OC. Total DNA was isolated from incubated samples using the DNA PowerSoil Isolation Kit (MoBio Laboratories Inc., Solana Beach, CA). Duplicates 100-mL aliquots of water were passed through a 0.22- μ m nylon filter (GE Water & Technologies, Trevose, PA). Filters were transferred into PowerBead tubes provided with the kit and then processed following the instructions of the manufacturer. The effects on the structure of the bacterial community were estimated by Amplified Ribosomal DNA Restriction Analysis (ARDRA). ARDRA is a method based on restriction endonuclease digestion of the amplified bacterial 16S rDNA. PCR amplification of 16S rDNA was carried out using the primer pair 63f/1387r (Table 3).

Target	Primer	Sequence (5'-3')
bacterial 16S rDNA	63f	CAGGCCTAACACATGCAAGTC
	1387r	GGGCGGWGTGTACAAGGC

Table 3 – Primers used for ARDRA analysis (Marchesi et al., 1998).

The PCR reaction mixture contained 25 μ L of RedTaq ReadyMix (Sigma–Aldrich Chemie GmbH, Munich, Germany), 0.5 μ M of each primer (Operon Biotechnologies, Inc., Huntsville, AL), 5–10 ng template DNA and water to a final volume of 50 μ L. The cycling was performed with the T3 DNA thermalcycler (Biometra GmbH, Göttinger, Germany) as follows: 94 °C (4 min) followed by 30 thermal cycles of 94 °C (30 s), 56 °C (30 s), 68 °C (60 s), and a final elongation step at 72 °C for 15 min. The size of the PCR products was verified by electrophoresis on a 1% agarose gel and visualized after staining with

SYBR Green I (Sigma–Aldrich). Aliquots of amplified 16S rDNA products (10 µL) were digested with 10 U of AluI and EcoRI (Sigma–Aldrich) in a total volume of 40 µL at 37 °C for 2 hours. Digested products were resolved by vertical non-denaturing 8% polyacrylamide gel electrophoresis and visualized by SYBR Green I staining. Data were computed with the software GelCompar II version 5.10 (Applied Maths, Sint-Martens-Latem, Belgium) to cluster the data and construct the similarity matrix to make comparisons of bacterial communities of the differently treated sample.

h. Microbial community structure by Denaturing Gradient Gel Electrophoresis (DGGE)

The structure and diversity of the bacterial community was estimated by a two-step nested-PCR denaturing gradient gel electrophoresis (DGGE) analysis. DGGE permits to see how bacterial sequences change over time and treatment. Prefiltered (0.45 µm) aliquots of water and water/sediment samples were passed through a sterile 0.22-µm nylon filter (GE Water & Technologies, Trevose, PA). Filters were transferred into PowerBead tubes provided with the kit PowerSoil DNA Isolation Kit (MoBio Laboratories Inc., Solana Beach, CA) and then processed following the instructions of the manufacturer. Total DNA was first amplified using the primer pairs P63f and P518r (Table 4) in a 50 µl reaction mixture consisting of 5-10 ng of DNA, 5 U of AmpliTaq DNA polymerase (Invitrogen, Carlsbad, CA), 10x reaction buffer, 4 mM MgCl₂, 0.5 mM of each dNTP, 0.8 µM of each primer and nuclease-free water. Reaction conditions were the following: denaturation at 94 °C for 5 min, followed by 31 cycles of denaturation at 94 °C for 60 s, annealing at 53 °C for 60 s, extension at 72 °C for 2 min, and final extension at 72 °C for 10 min. Amplicons were used for the second PCR using primer pairs P338f and P518r with the same cycler program (Table 4). 40-bp-long CG clamps were included at the 5' end of the forward primer.

Target	Primer	Sequence (5'-3')
bacterial 16S rDNA	P63f	CAGGCCTAACACATGCAAGTC
	P518r	ATTACCGCGGCTGCTGG
	P338f	ACTCCTACGGGAGGCAGCAG5

Table 4 – Primers used for DGGE analysis.

After quantification of amplified products, equal amounts of amplicons (250-300 ng) were loaded onto DGGE gel. Gel contained 8% (w/v) polyacrylamide gels (37.5:1 acrylamide:bisacrylamide) with a urea/formamide denaturing gradient of 40–60% (where 100% denaturant contains 7 M urea 8 and 40% v/v formamide). A 10-mL stacking gel containing no denaturants was added before polymerization was complete. Gels were run for 16 hours at 60 °C, with a constant voltage of 65 mV in 1 x TAE buffer. DGGE analysis was performed in DCode system (Bio-Rad Laboratories, Hercules, CA, USA). Bands were visualized after staining with GelRed (Biotum Inc., Hayward, CA). Band profiles were analyzed using the GelCompare II package (Applied Maths, Kortrijk, Belgium). Dendrograms were constructed using the DICE coefficients and were subjected to unweighted pair group method cluster analysis (UPGMA).

Bacterial community function

i. Bacterial viability by direct count

The relative abundance of viable bacteria in water samples was estimated by direct count, using fluorescent dyes. Cell viability was estimated using a cell viability kit (Live/Dead®, BacLight™), following the method proposed by Haglund et al. (2003). Two different fluorescent dyes were used, SYBR Green II, and propidium iodide, respectively as viability and membrane-compromised cell markers. Aliquots (1 mL) of water samples were incubated in the presence of SYBR Green II (1/10,000 dilution; Sigma-Aldrich, Germany) and propidium iodide (20 mM). After incubation, samples were filtered through a black polycarbonate filter (0.22 mm pore size) and viable (green) and dead (red) bacteria were enumerated by direct count using a Leica DM 4000B epifluorescence microscope at 1000 x magnification. Live cell abundance was calculated, as the number of live bacteria mL⁻¹, from the total cell abundance, obtained by DAPI counts, multiplied by % of live cells/live+dead.

j. Bacterial metabolic potential

The effects of OC on the metabolic potential of the autochthonous microorganisms were assessed by measuring mineralization of the pesticides

glyphosate and metolachlor in water samples of the CER irrigation canal. Samples of non-sterilized and sterilized water containing OC ($1.5 \mu\text{g mL}^{-1}$) were prepared as described above. Glyphosate and metolachlor were applied as water solutions using a mixture of unlabelled and ^{14}C -labelled compound in order to obtain a final concentration of $1 \mu\text{g a.i. L}^{-1}$. Unlabelled glyphosate (purity > 99%) and ^{14}C -glyphosate (*N*-phosphonomethyl-2- ^{14}C -glycine; radiopurity > 99%, specific activity $5.4 \text{ mCi mmol}^{-1}$) were purchased from Sigma-Aldrich Italia (Milan, Italy). Unlabelled metolachlor (purity > 96%) and ^{14}C -metolachlor (2-chloro-*N*-(2-ethyl-6-methyl-[U- ^{14}C]phenyl)-*N*-(2-methoxy-1-methyl-ethyl)acetamide; radiopurity > 99%, specific activity 13 mCi mmol^{-1}) were donated by Syngenta Crop Protection AG (Basel, CH). Treated water samples were incubated at 20°C on an orbital shaker (125 rpm) in the dark. Metolachlor and glyphosate mineralization was monitored by trapping the evolved $^{14}\text{CO}_2$ in vials containing 4 mL of a 1 M NaOH solution. The NaOH solution was replaced at sampling, facilitating flask aeration. Aliquots (1 mL) of NaOH solution were mixed with 4 mL of HiSafe 3 liquid scintillation cocktail (PerkinElmer, Boston, MA) and radioactivity quantified using a Wallac 1490 liquid scintillation counter (Wallac Oy, Turku, Finland). Samples were kept in the dark for 12 hours prior to analysis. Experiment was conducted in triplicate, and untreated samples (control) were included. Experiment was repeated with samples consisting of water/sediment mixture prepared as described above. Metabolic potential was expressed as the percentage of added glyphosate and metolachlor mineralized.

k. Nitrifying bacteria quantification by quantitative PCR (qPCR and qRT-PCR)

Quantitative PCR (Heid et al., 1996; Schmittgen, 2001) is a technique that permits a very accurate quantitative determination of DNA and RNA. It is based on detecting and quantifying a fluorescent probe or DNA-binding agent. By recording the amount of fluorescence at each cycle, it is possible to monitor the PCR reaction during the exponential phase, when the first significant increase in the amount of PCR product is correlated to the initial amount of target template. The more template is present at the beginning of the PCR reaction, the fewer cycles it takes to reach the point at which the fluorescent signal is first detected as being significantly greater than the background.

Total RNA was isolated by collecting cells onto a 0.22- μm filter as described for DNA isolation, except that filters were placed onto 2-mL centrifuge tubes containing 1 mL of Tri Reagent (Sigma–Aldrich). After homogenization by shaking, samples were incubated at room temperature for 5 min. Total RNA was separated from DNA and proteins by adding 0.2 mL of chloroform and centrifuging at 12000 g at 4 °C for 15 min. Finally, RNA was recovered from the aqueous phase by precipitation with isopropanol and redissolved in DEPC water. Remaining DNA was removed by the use of RNase-free DNase I (Sigma–Aldrich). RNA was reverse transcribed into complementary DNA (cDNA) and amplified using the SYBR Green Quantitative RT-PCR Kit (Sigma–Aldrich), following the manufacturer’s instructions. Expression level of genes involved in the bacterial nitrification process was performed by quantitative reverse transcriptase PCR (qRT-PCR), targeting ammonia-oxidizing 16S rRNA genes, functional ammonia monooxygenase (*amoA*) genes, and nitrite-oxidizing bacteria Nitrospira-like 16S-rRNA genes, using the primer pairs described in Table 5. Other than samples of the degradation study, triplicate water samples treated with increasing concentration of OC (0.02– 2 $\mu\text{g mL}^{-1}$) and incubated as described for the degradation study were included. Briefly, qRT-PCR was performed in 50 μL of the reaction mixture containing approximately 50 ng template RNA, 0.25 μM of each primer, 25 μL of SYBR Green Taq Ready Mix, 5 μL of the reference dye, 0.25 μL of Moloney Murine Leukemia Virus Reverse, and 12.5 μL of nuclease-free water. Thermocycling conditions were as follows: 2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 15 s at 95 °C and 1 min at 50 °C. The resulting samples were analyzed using an ABI Prism 7700 Sequence Detection System (Applied Biosystem Co., Foster City, CA). After quantification, amplified fragments samples were subjected to melting-curve analysis. A standard curve was generated by plotting cycle threshold values (Ct) against logarithmic-transformed amounts of target DNA obtained from 10-fold dilutions of DNA containing the target genes.

Target	Primer	Sequence (5’-3’)
bacterial 16S rDNA	1055f 1392r	ATGGCTGTCGTCAGCT ACGGGCGGTGTGTAC
<i>N. oligotropha</i> like <i>amoA</i> gene	<i>amoNo550D2f</i> <i>amoNo754r</i>	TCAGTAGCYGACTACACMGG CTTTAACATAGTAGAAAGCGG

ammonia-oxidizing bacterial 16S rDNA	CTO 189fA/B CTO 189fC RT1r	GGAGRAAAGCAGGGGATCG GGAGGAAAGTAGGGGATCG CGTCCTCTCAGACCARCTACTG
<i>Nitrospira</i> spp. 16S rDNA	NSR1113f NSR1264r	CCTGCTTTCAGTTGCTACCG GTTTGCAGCGCTTTGTACCG

Table 5 – Primers used in qPCR analysis. Respectively (Ferris et al., 1996; Dionisi et al., 2002; Hermansson et al., 2001; Harms et al., 2003).

Size of the total bacterial community, and of the ammonia-oxidizing bacteria, was estimated also by quantitative PCR (qPCR). Total bacteria were evaluated using a primer pair targeting the conservative bacterial 16S rDNA fragment. Ammonia-oxidizing bacteria were evaluated using two primers sets, including the ammonia-oxidizing bacterial 16S rDNA and the *Nitrosomonas oligotropha*-like *amoA* gene. Primers used in this study are described in Harms et al. (2003). Each 25 μ l qPCR reaction contained 2 μ l of DNA, 12.5 μ l of 2x TaqMan Universal PCR Master Mix (Applied Biosystems, CA), and 0.2 μ M of each primer. Thermocycling conditions were as follows: 2 min at 50 °C, 10 min at 95 °C, cycles of 15 s at 95 °C and 1 min at 60 °C. Reactions were performed using an ABI Prism 7700 Sequence Detection System (Applied Biosystems). Gene copies were estimated by comparison of cycle threshold values obtained from known amounts of DNA. All analyses were conducted in triplicate.

1. Bacterial isolates and bioremediation tests

Environmental samples were used as a source of inoculum for enrichments on OC. Enrichment cultures were done in a definite minimal salt medium (MSM) containing (g L⁻¹): KH₂PO₄, 0.4; K₂HPO₄, 1.6; CaSO₄ 2H₂O, 0.1; MgSO₄ 7H₂O, 1.0; (NH₄)SO₄, 2.0. A volume of 1 mL was added to 250-mL flasks containing 50 mL of MSM and 50 mg L⁻¹ of analytical grade OC. Flasks were incubated at 25 °C with shaking (250 rpm). Enrichment cultures were sub-cultured six times by transferring 0.5 mL to a fresh MSM containing OC. Pure cultures were isolated from the enrichments by streaking out on solid MSM supplemented with 50 mg L⁻¹ OC. Colonies were picked and screened for their ability to mineralize OC. After growing overnight in LB broth, cells were harvested by centrifugation at 10,000 g for 10 min and washed twice with sterile phosphate buffer saline (PBS). The

pellet was resuspended in PBS to the initial density of approximately 4×10^6 cell mL^{-1} . For each single colony, 0.5 mL of inoculum was transferred to biometers flask containing MSM and a mixture of unlabeled and ^{14}C -OC to give a final concentration of $30 \mu\text{g mL}^{-1}$. Biometers were incubated for 10 days at 25°C with shaking (250 rpm). Trapped $^{14}\text{CO}_2$ was measured as described above. Two strains which showed higher potential for OC mineralization were selected for the bioremediation study. Bacterial strains were identified by sequencing a PCR-amplified fragment of the bacterial 16S rDNA. Genomic DNA was isolated using the commercial kit UltraClean Microbial DNA Isolation (MoBio Laboratories Inc., Solana Beach, CA) following the manufacturer's instructions. Isolated DNA was amplified by PCR using the universal primers as described in Willems and Collins (1996). PCR products were purified using the QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany) and DNA sequencing was performed by cycle sequencing using the DyeDeoxy Terminator Cycle Sequencing Kit and an ABI Prism DNA Sequencer (Applied Biosystem, Foster City, CA, USA). DNA sequences of the genomic 16S rDNA were compared with existing sequences using BLAST (<http://www.ncbi.nlm.nih.gov>) and submitted to GenBank under the Accession Numbers GU065286 and GU065287.

Bacterial strains were used to assess their potential to mineralize OC in water and water/sediment samples. The experiment was conducted in biometer flasks containing 50 mL of samples and OC (10 g mL^{-1}). Flasks were separately inoculated with one or the combination of the isolated strains following the procedure described above. Trapped $^{14}\text{CO}_2$ was measured by LSC.

The efficiency of a Patent Pending formulation, using the fungus *Phanerochaete cryosporium*, strain DSMZ 1547, was tested for bioremediation of Tamiflu in the municipal wastewater treatment plant (WWTP) of Bologna. Samples of the ASML and EW (100 mL) were transferred into 250-L flasks under aseptic conditions. Analytical grade OC was applied to flasks to give a final concentration of $10 \mu\text{g mL}^{-1}$. After adding the fungus formulation, flasks were sealed and incubated for 30 days on an orbital shaker (125 rpm) at 20°C in the dark. At selected intervals, triplicate samples were taken for chemical and microbial analysis.

3.1.3 Study cases in different water ecosystems

3.1.3.1 Irrigation canal in Emilia Romagna

Experimental site, sampling and microcosm set up

Water and sediment samples were collected in April 2006 from the irrigation canal Canale Emiliano Romagnolo (CER) in proximity of Medicina (44°27'59'' N, 11°42'25'' E) (Figure 8). CER is a 133 km long canal that receives water from the River Po, runs from Salvadonica di Bondeno (Ferrara) downs to Donegaglia di Bellaria (Rimini) before emptying in the Uso river.



Figure 8 – View of the irrigation canal Canale Emiliano Romagnolo (CER).

Samples were collected manually by immersing 2-L sterile glass bottles approximately 10 cm below the water surface. Collected samples were transported to the laboratory within 2 hours from sampling and were kept at 4 °C in the dark. Prior to use, water samples were left at 20 °C overnight. A portion of the collected water was sterilized by autoclaving for 1 hour at 121 °C and 103 kPa. Some of the physico-chemical properties of the collected water and sediment are given in Table 6.

<i>Water</i>									
pH	DO (mg L ⁻¹)	DOC (mg L ⁻¹)	HCO ₃ ⁻ (mg L ⁻¹)	F ⁻ (mg L ⁻¹)	Cl ⁻ (mg L ⁻¹)	NO ₂ ⁻ (mg L ⁻¹)	NO ₃ ⁻ (mg L ⁻¹)	PO ₄ ³⁻ (mg L ⁻¹)	SO ₄ ²⁻ (mg L ⁻¹)
8.3	13.1	4.7	158.0	0.17	28.5	0.07	13.2	<0.02	83.8

<i>Sediments</i>			
Sand	Silt	Clay	Organic Carbon
7.3%	38.9%	53.8%	2.8%

Table 6– Physico-chemical properties of water and sediment from irrigation canal Canale Emiliano Romagnolo (CER). DO: dissolved oxygen. DOC dissolved organic carbon.

Water samples (80 mL) were transferred into 250-mL sterile Erlenmeyer flasks under aseptic conditions. Samples were treated with oseltamivir carboxylate (OC) dissolved in 50 mM NaH₂PO₄ to give a final concentration of 1.5 µg mL⁻¹. Flasks were sealed, and samples were incubated at 20 °C on an orbital shaker (125 rpm) in the dark. At selected time intervals, duplicate 1-mL aliquots were taken for analytical or microbiological analysis. All operations were conducted under sterile conditions. Control samples, consisting of untreated water and samples containing 5% (w/v) of sterilized or non-sterilized sediments taken from the same irrigation canal, were included. Sediments were air-dried and passed through a 5-mm sieve. The whole experiment was conducted in triplicate.

Chemical analysis

Degradation of OC was assessed by HPLC, as described in paragraph a.

Microbial analysis

Bacterial abundance (e), viability (i), phylogenetic composition using the probes for the identification of the bacterial taxa: *Bacteria*, *α-Proteobacteria*, *β-Proteobacteria*, *γ-Proteobacteria*, *Planctomycetes*, *Cytophaga-Flavobacterium*, *Firmicutes* (f), and metabolic potential (j) were assessed as described in the mentioned paragraphs.

3.1.3.2 River Po and Venice Lagoon

Experimental site, sampling and microcosm set up

Samples of water and sediment were collected in November 2007 from the River Po (Occhiobello, Italy) and the Venice Lagoon (in proximity of St. Mark's Square, Venice, Italy) (Figure 9).

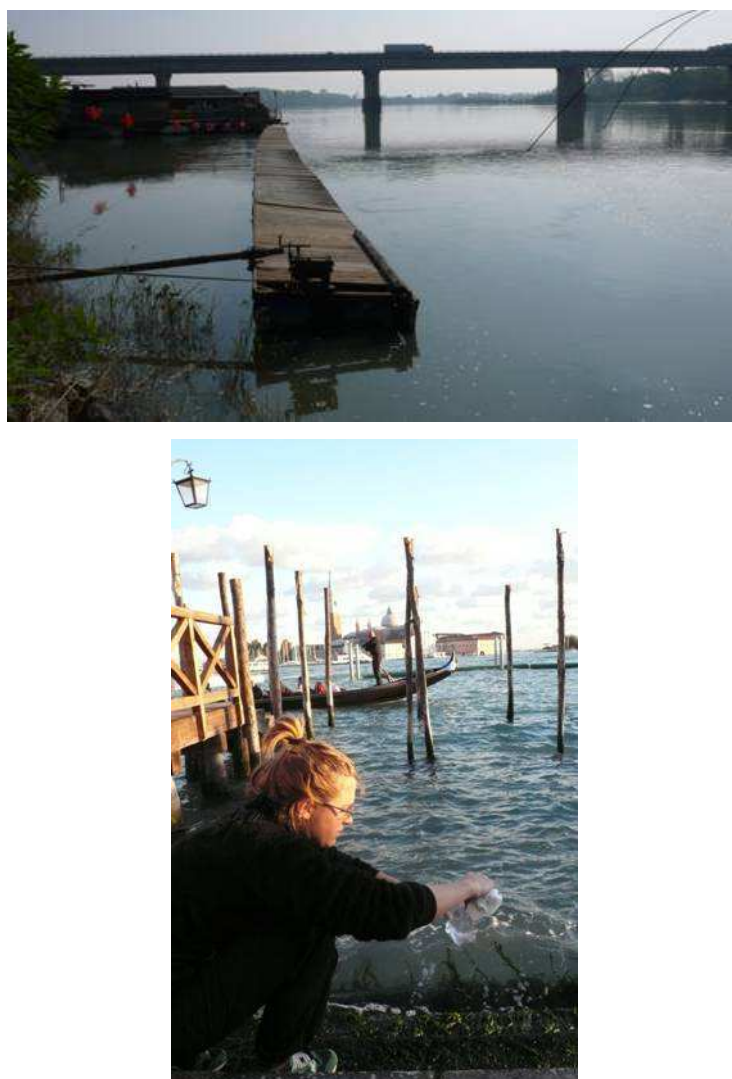


Figure 9 – Sampling points in River Po (above) and Venice Lagoon (below).

In both sites, samples were collected manually by immersing 2-L sterilized glass bottles approximately 10 cm below the water surface. Sediments were collected using a sterilized stainless steel corer. Samples were transported to the laboratory within 2 hours from sampling and kept at 4 °C in the dark for no longer than three days. Prior to use, water samples were left at 20 °C overnight. Sediments were left to dry at room temperature for two days, homogenized by passing through a 4-mm sieve and the remaining water content was determined gravimetrically. A portion

of the collected water and sediments was sterilized by autoclaving for 1 hour at 121 °C and 103 kPa on three successive days. Selected physico-chemical and microbiological properties of the collected water and sediments are given in Table 7.

	River Po	Venice Lagoon
<i>Water</i>		
pH	6.91	7.60
Dissolved Organic Carbon (mg L ⁻¹)	1.49	2.29
Suspended particulate matter (mg L ⁻¹)	62.16	99.77
Bacterial number (log cell number mL ⁻¹)	5.82	5.91
NH ₃ (mg L ⁻¹)	5.71	6.82
Salinity (psu)	<1	27.22
<i>Sediments</i>		
pH	7.10	7.71
Sand (%)	91.45	94.09
Silt (%)	4.45	5.12
Clay (%)	4.10	0.79
Total carbon (%)	2.68	5.68
Total nitrogen (%)	0.17	0.21
Bacterial number (log cell number g ⁻¹)	7.91	7.48

Table 7 – Selected properties of water and sediment of the River Po and Venice Lagoon.

For the degradation study, microcosms were prepared by transferring water (500 mL) and sediments (5 and 10% w/v) from the aquatic ecosystems into 1-L flasks under aseptic conditions. Analytical grade OC (chemical purity > 98%) was applied to each sample to give a final concentration of 20 µg L⁻¹. Finally, flasks were sealed and incubated for 21 days on an orbital shaker (125 rpm) at 20 °C in the dark. Untreated flasks were included as control for microbiological analysis. At selected intervals, duplicate samples were taken for chemical and microbial analysis.

Chemical analysis

Degradation and Mineralization of OC were assessed as described in paragraph b and c, bioavailability and sorption isotherms of OC as in paragraph d.

Microbial analysis

Microbial community structure by ARDRA, and the nitrifying bacteria quantification by qRT-PCR were assessed as described in paragraphs g and k.

3.1.3.3 Lake Biwa and River Furukawa

Experimental site, sampling and microcosm set up

Samples of water and sediments of Lake Biwa (34° 59.81'N, 135° 53.68'E) and River Furukawa (34° 54.10'N, 135° 44.87'E) were collected in Japan, on January 2009 and shipped to Europe in a refrigerated container ensuring a constant temperature of 4 °C within. Sediments of the two Japanese water bodies were left to dry at room temperature and homogenized by passing through a 4-mm sieve.

Lake Biwa is the largest lake and the greatest water resource in Japan. It supplies municipal and industrial water to 14 million residents around and downstream of the lake. It has a surface area of 670 km² with a maximum depth of 103.6 m. River Furukawa is one of Japan's first-class rivers, it is a branch of River Yahagi, which flows from Nagano Prefecture's Mount Ōkawairi, through Gifu Prefecture, and enters Mikawa Bay from Aichi Prefecture.

Selected physico-chemical properties of the collected samples are given in Table 8.

	Lake Biwa	River Furukawa
pH	7.3	7.1
Chemical Oxygen Demand (mg L ⁻¹)	3.3	3.7
Conductivity (mS/M)	15.6	18.5
Turbidity	20.0	23.1
Total nitrogen (mg L ⁻¹)	0.29	1.4
Total phosphorus (mg L ⁻¹)	0.01	0.07

Table 8 - Selected properties of Lake Biwa and River Furukawa waters.

Microcosms for the degradation study were set up by transferring water (250 mL) and sediments (5% w/v) from the two Japanese aquatic ecosystems into 1 L flasks under aseptic conditions. Analytical grade OC was applied to each sample to give a final concentration of 40 µg L⁻¹, and samples were incubated for 40 days.

Chemical analysis

The same procedure, described previously in paragraphs b and c, was followed for degradation and mineralization analysis.

Microbial analysis

Microbial community structure was assessed by ARDRA and DGGE following the procedure described previously, in paragraph g and h. Nitrifying bacteria quantification was conducted by qPCR (paragraph k), and bacterial strains were isolated and tested for bioremediation (paragraph l).

3.1.3.4 Wastewater Treatment Plant of Bologna

Experimental site, sampling and microcosm set up

Samples of activated-sludge-mixed liquor (ASML) were obtained from the municipal wastewater treatment plant (WWTP) of Bologna, Italy (44° 33.09'N, 11° 21.48'E) in May 2009 (Figure 10). The selected WWTP has a surface of 150,000 m² and a capacity of up to 900,000 population equivalent and included the use of pressure swing technology with partial recycling of dewatered sewage sludges for energy production. After chlorine oxidation and microfiltration, treated effluent is discharged into the Navile canal. The average hydraulic retention period of the plant is 4-5 hours.



Figure 10 – Air photo of the municipal wastewater treatment plant (WWTP) of Bologna.

Samples of effluent water (EW) were also included in the study. Sampling operations were conducted in May 2009 using aseptic techniques. Samples were stored at 4 °C for no longer than 2 days. Prior to use, all the samples were left at 20 °C overnight. Selected properties of the samples used in this study are summarized in Table 9.

	Bologna ASML	Bologna EW
pH	7.1	7.8
Chemical Oxygen Demand (mg L ⁻¹)	35.4	36.2
Biological Oxygen Demand (mg L ⁻¹)	10.7	7.0
Total Suspended Solids (mg L ⁻¹)	14.5	10.6
N-NH ₃ (mg L ⁻¹)	7.6	11.2

Table 9 - Selected properties of activated-sludge-mixed liquor (ASML) and effluents (EW) of the wastewater treatment plant of Bologna.

Chemical analysis

The degradation study was performed by transferring samples from the WWTP into 1 L flasks under aseptic conditions. Analytical grade OC was applied to each sample to give a final concentration of 40 µg L⁻¹, and samples were incubated for 40 days. The same procedure described previously in paragraphs b and c was followed for degradation and mineralization analysis of the WWTP samples.

Microbial analysis

Isolated bacterial strains were tested for mineralization capability in ASML and in EW samples (paragraph l).

3.2 CHAPTER 2 – Fate and removal of common use antibiotics in WWTP

3.2.1 Experimental site, sampling and microcosm set up

A series of laboratory studies was conducted to evaluate the degradation of three antibiotics, erythromycin, sulfamethoxazole, and ciprofloxacin in the same wastewater treatment plant described in paragraph 3.1.3.4. The efficiency of a Patent Pending formulation, using the fungus *P. cryosporium* strain DSMZ 1547, was tested for bioremediation in the wastewater from the studied pharmaceuticals.

All antibiotics were of analytical grade (> 98 %) and were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Samples of the incubation study were prepared by transferring ASML or EW (100 mL) into 250-L flasks under aseptic conditions. Analytical grade pharmaceuticals were separately applied to each flask to give a final concentration of 10 µg mL⁻¹. After adding the fungal formulation, flasks were sealed and incubated for 30 days on an orbital shaker (125 rpm) at 20 °C in the dark. At selected intervals, triplicate samples were taken for chemical and microbial analysis.

3.2.2 Chemical analysis

Samples were extracted by solid phase extraction and analyzed by liquid chromatography/electro spray tandem mass spectrometry. Analysis of the three pharmaceuticals were performed using a triple stage quadrupole MS/MS TSQ Quantum Ultra EMR (Thermo Fisher Scientific, San Jose, CA, USA) coupled with an Accela LC pump (Thermo Fisher Scientific, San Jose, CA, USA) and a PAL HTC autosampler (CTC Analytics AG, Zwingen, Switzerland). Samples (20 µL) were loaded onto a Hypersil GOLD aQ TM column (50 mm x 2.1 mm ID x 5 µm particles, Thermo Fisher Scientific, San Jose, CA, USA). Elution conditions were programmed as follows: water/methanol (90/10) for 1 min, then composition was changed to water/acetonitrile/methanol (30/10/60) for 8 min and then the column was washed for 9 min by acetonitrile/methanol (60/40). Heated electrospray in positive ion mode was used for ionisation of target compounds. Key parameters were the following: ionisation voltage 3.5 kV, sheath gas 50 and auxiliary gas 35 arbitrary units, vaporiser temperature 100 °C, capillary temperature 325 °C, collision gas was argon at 1.5 mL min⁻¹. Both first and third quadrupole were operated at resolution 0.7 FWHM. Three SRM transitions were monitored for each analyte. Samples were quantified using internal standards. Prior to analysis, samples were kept in the dark for 12 hours. The whole incubation study experiment was conducted in triplicate. Data of the degradation study were analyzed by analysis of variance. Means were separated by Fisher's least significant difference (LSD) and significant differences were detected at the P = 0.05 level.

3.2.3 Microbial analysis

Triplicates samples prepared as for the incubation study were used for microbiological analysis. Growth of the introduced bioremediation fungus was monitored by quantitative PCR (qPCR) using the primer pair Cu1F/Cu2R targeting the laccase gene (Luis et al., 2004). Granules used for amending ASML and EW samples were periodically removed and total DNA was isolated using the CTAB method (Doyle and Doyle, 1990). Briefly, triplicates granules were removed from each incubated sample, dried at 40 °C for 2 hours, vortexed for 5 min to remove adhering organic particles and air-flushed by high-pressure air. Surface-cleaned granules were transferred to a 2-mL microcentrifuge tube containing 500 µL of CTAB buffer and glass beads (425-600 µm; Sigma-Aldrich). After vortexing for 2 min, tubes were incubated at 65 °C for 15 min, and an equivalent volume of chloroform:isoamyl alcohol (24:1) was added to tubes. Tubes were gently shaken and centrifuged at 10,000 x g for 5 min before the addition of 2/3 volume of isopropanol/ammonium acetate to precipitate the DNA. The pellet was rinsed with 70% ethanol, air dried, resuspended in 100 µL of TE buffer and used for qPCR. Each 25 µL qPCR reaction contained 2 µL of DNA, 12.5 µL of 2× TaqMan Universal PCR Master Mix (Applied Biosystems, CA), and 0.2 µM of each primer. Thermocycling conditions were as follows: 2 min at 50 °C, 10 min at 95 °C, cycles of 15 s at 95 °C and 1 min at 60 °C. Reactions were performed using an ABI Prism 7700 Sequence Detection System (Applied Biosystems). Gene copies were estimated by comparison of cycle threshold values obtained from known amounts of DNA. All analyses were conducted in triplicate. Structure of the bacterial and fungal communities of incubated samples was investigated by PCR- denaturing gradient gel electrophoresis (DGGE) analysis. Prefiltered (0.45 µm) aliquots of ASML and EW samples were passed through a sterile 0.22-µm nylon filter (GE Water & Technologies, Trevose, PA). Filters were transferred into PowerBead tubes provided with the UltraClean Soil DNA Isolation Kit (MoBio Laboratories Inc., Solana Beach, CA) and then processed following the instructions of the manufacturer. For bacterial PCR-DGGE, total DNA was amplified using the primer pair GC-968f/1401r targeting the V6-V8 region of the 16S rDNA (Heuer et al., 1999). Amplification reactions were performed in a 50 µL reaction mixture consisting of 5-10 ng of DNA, 1.25 U of BioTaq DNA polymerase (Bioline Abcys, Paris, France), 10× PCR buffer, 2.5 mM MgCl₂, 200 µM of each dNTP, 0.5 µM of each primer, 250 ng µL⁻¹ bovine

serum albumin and nuclease-free water. Reaction conditions were the following: denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 97 °C for 45 s, annealing at 58 °C for 60 s, extension at 72 °C for 45 s and final extension at 72 °C for 5 min. For fungal PCR, a semi-nested approach targeting the internal transcribed spacer region (ITS) was adopted. Primer pairs and PCR conditions are described elsewhere (Crouzet et al., 2010). Equal amounts of amplicons (800 ng) were loaded onto DGGE gel. Gel contained 8% (w/v) polyacrylamide gels (37.5:1 acrylamide:bisacrylamide) with a urea/formamide denaturing gradient of 45 – 65% for bacteria and 25–55% for fungi (where 100% denaturant contains 7 M urea and 40% v/v formamide). Gels were run for 16 hours at 60 °C, with a constant voltage of 140 V for bacteria and 70 V for fungi. DGGE was performed in DCode system (Bio-Rad Laboratories, Hercules, CA, USA). Bands were visualized after staining with Gel Star Nucleic acid gel stain and digitized using the Versa Doc Imaging System (Bio-Rad). Band profiles were analyzed using the GelCompare II package (Applied Maths, Kortrijk, Belgium). Dendrograms were constructed using the DICE coefficients and were subjected to unweighted pair group method cluster analysis (UPGMA).

3.3 CHAPTER 3 – Veterinary Pharmaceuticals

3.3.1 Experimental site, sampling and microcosm set up

Soil from an olive tree grove in Aranjuez, in central Spain, was sampled in 2008. The agricultural soil is routinely amended with livestock residues from a pig farm. Two sets of microcosms were arranged by adding to the soil the veterinary drugs oxibendazole, to give a final concentration of 5 µg mL⁻¹, and doramectin, with 20 µg mL⁻¹ final concentration. Samples were incubated at 20 °C on an orbital shaker (125 rpm) in the dark. After respectively 21 and 23 days of incubation, soil aliquots were used for analytical and microbiological analysis. All operations were conducted under sterile conditions. Control samples, consisting of untreated soil, were included.

3.3.2 Microbial analysis

In order to investigate the effects of oxibendazole and doramectin on the phylogenetic structure of the soil bacterial community, filters with cells, fixed as

described previously in paragraph (f), were analysed by Fluorescence *In Situ* Hybridization. Phylogenetic probes were applied for the identification of the following bacterial taxa: *Archaea*, *Bacteria*, *α -Proteobacteria*, *β -Proteobacteria*, *γ -Proteobacteria*, *δ -proteobacteria* Sulfate-Reducing Bacteria, *ε -proteobacteria*, *Cytophaga-Flavobacterium*, *Actinobacteria*, *Firmicutes*.

Furthermore, because of the importance of nitrate-reducing bacteria in the nitrogen cycle, the effects of the two veterinary drugs on this functional group were investigated. Denitrification involves the reduction of nitrate, via nitrite and nitric oxide, to nitrous oxide or dinitrogen gas by a respiratory process under oxygen-limiting conditions. In particular in this work, the nitrate reductase gene *narG*, was of special interest to provide information on the denitrifying bacteria in the environment. Denitrification was induced by adding sodium nitrate (10 mM) to soil, and simultaneously shifting to O₂-limited conditions by sealing the sample with a paraffin layer and avoiding shaker rotation.

The presence of the *narG* gene in soil samples was checked by PCR amplification from extracted DNA, prior to analysis of gene expression. Total DNA was isolated from incubated samples using Ultra Clean Soil DNA Kit (MoBio Laboratories, Inc. Solana Beach, CA, USA). A set of partially degenerated primers for the amplification of the *narG* gene (Table 10), in yet uncultivated bacteria in the environment, was used for PCR and qRT-PCR assays. Total RNA was isolated from soil using the FastRNA® Pro Soil-Direct Kit (MP Biomedicals, Solon, OH). Samples were processed following the instructions of the manufacturer. Reverse transcriptase was obtained by utilization of the iScript™cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA), and amplification was obtained by the use of SYBR Green PCR mix (iTaQ™ SYBR® Green Supermix with ROX, Bio-Rad). Conditions described in (López-Gutiérrez et al., 2004) were used for the qRT-PCR assay.

Target	Primer/Probe	Sequence (5'-3')
<i>narG</i> gene	1960m2f	TA(CT)GT(GC)GGGCAGGA(AG)AAACTG
<i>narG</i> gene	2050m2r	CGTAGAAGAAGCTGGTGCTGTT

Table 10 – *narG* primers and probe used in the study for qPCR and FISH analysis (López-Gutiérrez et al., 2004).

The sequence of the *narG* primer 2050m2r was used to build a specific fam-labelled probe, for the detection of the *narG* gene by Fluorescence *in Situ* Hybridization in the incubated and non-incubated soil samples (paragraph f). Conditions described previously were used for the FISH analysis (Martin et al., 2008). Percentages of *narG*-harbouring cells were calculated based on the total number of cells stained with DAPI.

Isolation of bacterial strains

Bacterial strains, able to grow on the veterinary drugs as sole carbon source, were isolated from the soil sample by enrichment culture in minimal medium MB (K_2HPO_4 : 1.6 g L⁻¹; KH_2PO_4 : 0.4g L⁻¹; $\text{CaSO}_4 \times 2 \text{ H}_2\text{O}$: 0.1 g L⁻¹; $\text{MgSO}_4 \times 7 \text{ H}_2\text{O}$: 1.0 g L⁻¹; $\text{FeSO}_4 \times 7 \text{ H}_2\text{O}$: 0.02 g L⁻¹; $(\text{NH}_4)_2\text{SO}_4$: 2 g L⁻¹; Agar: 15 g L⁻¹) supplemented with doramectin (100 µg mL⁻¹) and oxibendazole (100 µg mL⁻¹). DNA from each isolate was extracted using the method described in Casas et al. (1995). Amplification of rDNA gene was performed by PCR using the universal primer ARI (5' GAGAGTTTGATCCTGGCTCAGGA 3'), and reverse pH (5' AAGGAGGTGATCCAGCCGCA 3'). The amplified fragments were sequenced and compared with sequences available in the GenBank/EMBLdatabases (<http://www.ncbi.nlm.nih.gov>).

4. RESULTS AND DISCUSSION

4.1 CHAPTER 1 – The antiviral drug Tamiflu

4.1.1 Irrigation canal in Emilia Romagna

Chemical analysis

Degradation of OC

Degradation of OC in water and water/sediment samples over the course of the 36-day incubation period is shown in Figure 11. Degradation of OC in water did not adequately fit the first-order model ($r^2 \leq 0.80$). After a rapid decrease, OC concentrations slowly decreased during the remaining incubation period. Approximately 65% of the applied amount degraded in water samples within 36 days. These findings suggested that degradation of OC in water is a complex process, not simply described by the linear model. OC was less persistent in samples containing sediments (5% w/v). In contrast to water samples, the linear model gave a strong fit ($r^2 > 0.96$) to the degradation of OC in water/sediment mixtures. The estimated half-life of OC in the water/sediment microcosm was 21 days. Chemical analysis revealed that approximately 5% of the applied OC was degraded within 36 days in sterilized water samples (Figure 11). Similar values were observed in the sterilized water/sediment mixture (data not shown). This information provides supporting evidence that OC degradation was mainly driven by microbial processes. Considering the size of the cultivable bacterial population ($7.9 \pm 0.28 \log \text{CFUs g}^{-1}$ air-dried sediments), the effect of sediments is compatible with the increasing microbial abundance and metabolic potential of the microcosm. Enhanced biodegradation of xenobiotics in the presence of sediments has been reported for a number of compounds, including pesticides and antimicrobials (Walker et al., 1984; Pritchard et al., 1987; Kim et al., 2004). This effect could result from a greater number of microorganisms on the surface of sediment particles, an increased activity of microorganisms in the presence of sediments due to greater availability of nutrient, or an ability of sediments to concentrate chemical through sorption (Walker et al., 1984). Under some circumstances, abiotic processes (i.e. hydrolysis and photolysis) can have an important role in the degradation of pharmaceuticals in water (Liu et al., 2001).

The present work was conducted in the dark, and consequently it cannot be excluded that abiotic processes would have a greater importance in the degradation of OC under normal light conditions.

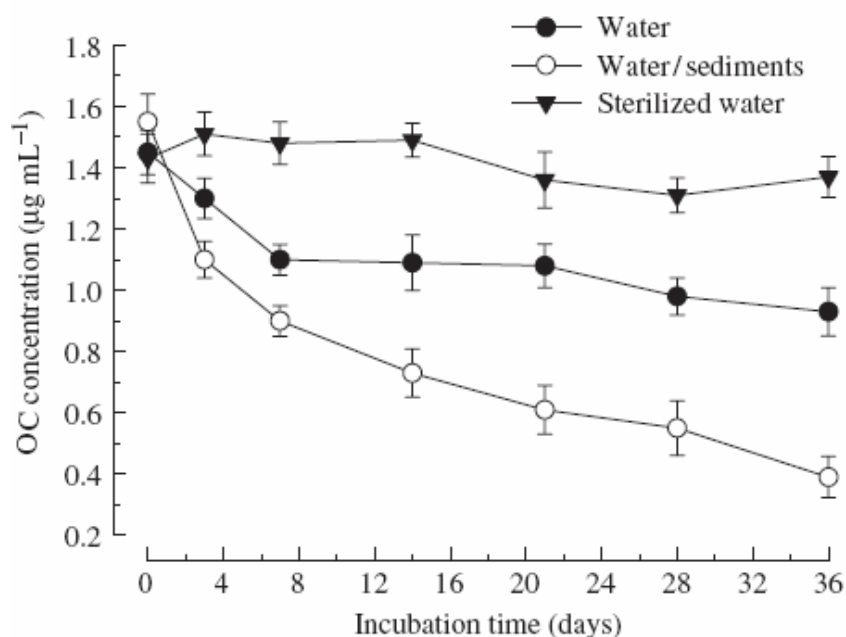


Figure 11 - Degradation of oseltamivir carboxylate (OC) in water and water/sediment samples of the irrigation canal Canale Emiliano Romagnolo (CER). Bars represent standard deviations of the means.

Presented results indicate that OC is moderately persistent in the water of the CER irrigation canal. This appears to be mainly due to the reduced intensity of microbial degradation processes as further evidenced by results of the glyphosate and metolachlor mineralization experiment. The low biodegradability of a wide number of pharmaceuticals in surface water has been previously documented (Alexy et al., 2004).

Microbial analysis

Bacterial abundance

The size of the bacterial population of untreated water samples (control) remained approximately constant during the whole 36-day incubation period, except a transient decrease at the end of the second week of sample incubation. Addition of the antiviral drug OC ($1.5 \mu\text{g mL}^{-1}$) led to a significant decrease in the number of bacteria during the first half of the incubation period. The highest decrease was observed in samples containing the antiviral drug. In the remaining period, the number of bacteria remained significantly higher in samples treated with OC than

in the control (Table 11). Even though pharmaceuticals are specifically designed to perform some sort of biological effect, the direct and indirect effects of pharmaceuticals on non-target organisms have received little attention (Accinelli et al., 2006). OC is a selective inhibitor of influenza virus neuraminidase, an enzyme involved in the release of new virus particles from infected cells (Eisenberg and Cundy, 1998). Based on its specific mode of action and in contrast to antimicrobials, which are active against bacteria, no direct toxic effect of OC on water microorganisms would be expected. However, this does not exclude the possibility that OC may have indirect effects on non-target microorganisms. This phenomenon has been evidenced for a wide number of compounds, including pharmaceuticals (Kümmerer et al., 2000; Engelen et al., 1998; Busse et al., 2001).

Incubation time (days)	Bacteria abundance (log cell number mL ⁻¹)	
	Control	OC treated
0	6.24±5.64	6.12±5.35
3	5.95±4.28	5.22±5.05
7	5.83±4.92	5.37±4.51
14	6.53±5.46	5.78±4.81
21	6.27±4.93	6.92±6.22
28	6.07±5.28	6.42±5.45
36	6.01±4.97	6.55±5.87

Table 11 – Abundance of the bacteria population (log cell number mL⁻¹) of untreated water (control) and water samples treated with oseltamivir carboxylate (OC) during the incubation period. Values are means of three replicates ± standard deviations.

Bacterial community structure and vitality

The addition of OC led to an initial decrease in the number of *Bacteria* cells, detected by the EUB probes. However, at day 21 a significant peak ($p < 0.01$) in the *Bacteria* cell number was observed in the OC-treated samples (Figure 12 A). This trend was observed also ($p < 0.01$) for α -*Proteobacteria*, β -*Proteobacteria* and γ -*Proteobacteria* (Figure 12 B, C, D). In particular, the β -*Proteobacteria* group was the most abundant and constituted 40% of the *Bacteria* domain, suggesting an active role in the OC degradation. The other bacterial groups investigated by FISH were not significantly affected by the presence of OC, and represented about 1-2% of the *Bacteria* domain. In particular *Cytophaga-Flavobacterium* was the relatively most abundant group (the average number mL⁻¹

during the experimental period was $1.39\text{E}+04 \pm 4.02\text{E}+03$) followed by *Actinobacteria* ($2.8+03 \pm 1.67+03$) and finally *Planctomycetes* ($1.22\text{E}+04 \pm 7.3+03$). The results of the bacterial community analysis show that the decrease at day 14 in bacterial abundance of the main phylogenetic groups in presence of OC was transient. This initial negative effect was subsequently offset by a significant increase in their presence and presumably activity at day 21. This hypothesis is confirmed by the fact that about 65% of the OC applied was degraded in 36 days in water samples, while just 5% was degraded in the sterilized water, as shown previously. Consequently the overall results confirmed the key role of the bacterial community in OC degradation, and suggested which bacterial groups, i.e. α -*Proteobacteria*, γ -*Proteobacteria* and above all β -*Proteobacteria* could be directly involved. These results encourage the performance of further studies to better investigate the bacterial metabolism (and/or co-metabolism) of this compound and the formation of its transformation products prior to its possible mineralization. The presence/absence of bacterial populations with a natural attenuation capacity versus pharmaceuticals is a crucial factor in assessing their actual environmental fate in aquatic ecosystems.

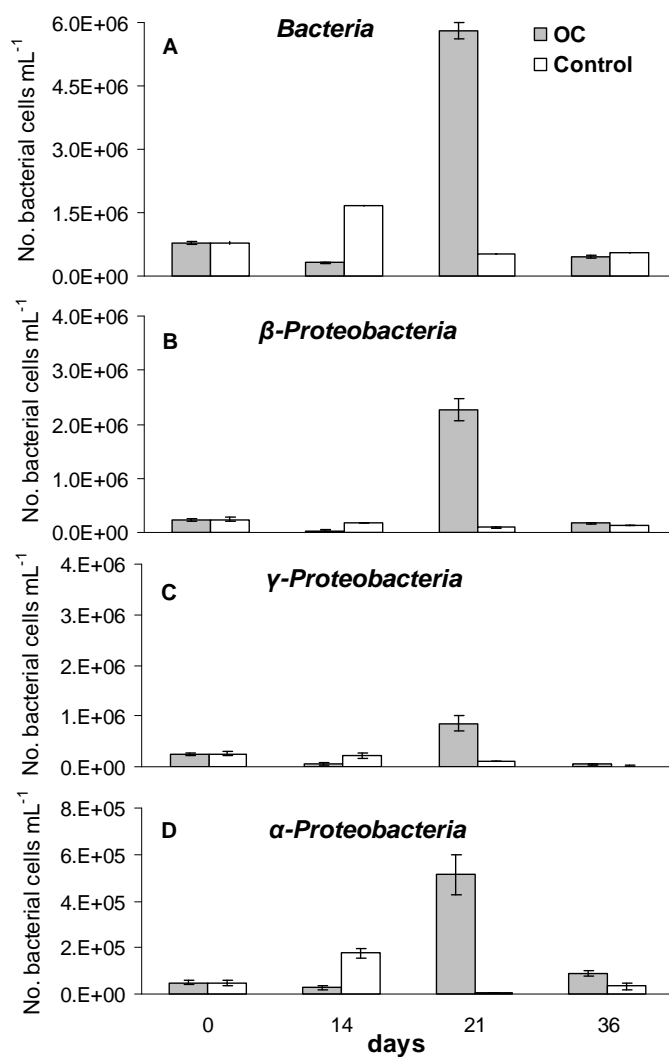


Figure 12 - Bacterial community structure detected by FISH in surface water at different times: 0, 14, 21 and 36 days, in presence of oseltamivir carboxylate (OC) and control. Vertical bar represent standard errors.

FISH results are in line with the transient decrease in live cell vitality (No. live bacteria mL^{-1}) at day 14, followed by a significant increase at day 21 in OC-treated samples (Figure 13).

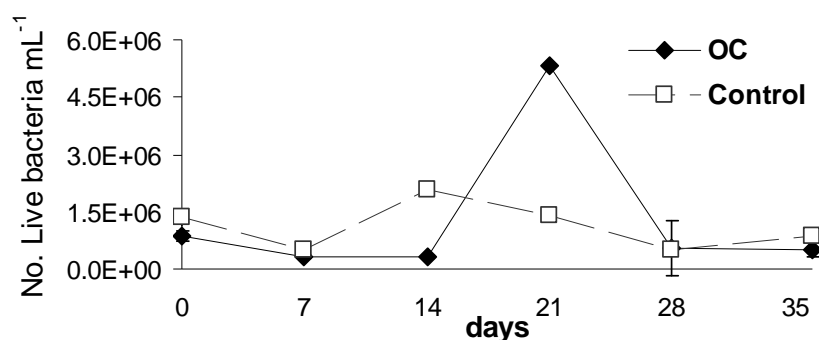


Figure 13 - Live cell abundance (No. live bacteria mL⁻¹) at different sampling times in OC-treated and Control in surface water samples.

Metabolic potential of water microorganisms

General microbial activity was estimated from the mineralization of radiolabelled glyphosate and metolachlor. These two herbicides were chosen as models of chemicals which are degraded by a wide number of microorganisms (Accinelli et al., 2005). Representative mineralization values of glyphosate and metolachlor, expressed as ¹⁴CO₂ evolution, in water and water/sediment samples are shown in Figure 14. As expected, mineralization of glyphosate and metolachlor proceeded without a lag phase, thus confirming that these two chemicals are degraded by a variety of microorganisms and that microbial adaptation is not strictly necessary.

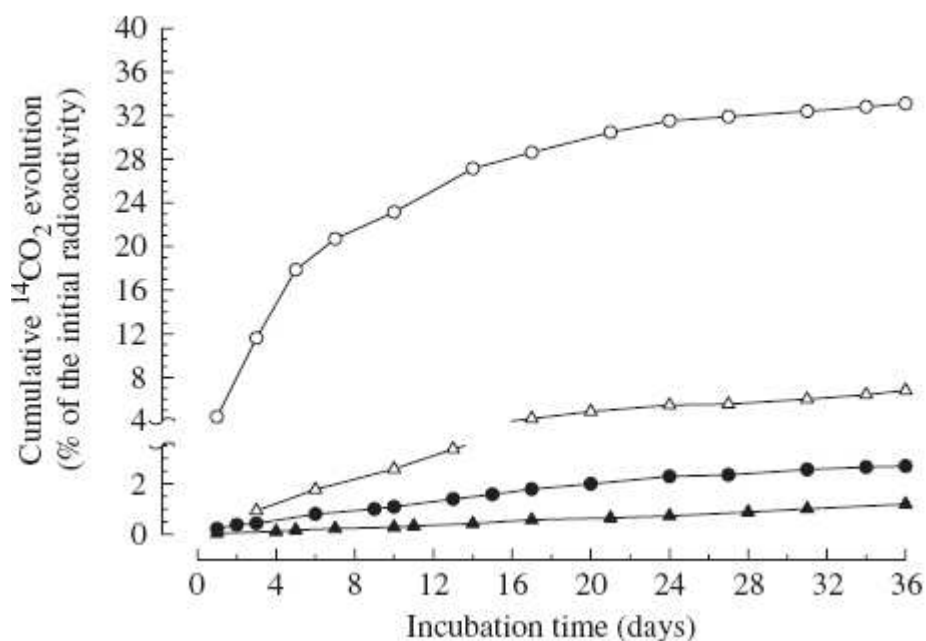


Figure 14 - Glyphosate (circles) and metolachlor (triangles) mineralization in water (empty symbols) and water/sediment (full symbols) samples.

During the 36-day incubation period, cumulative $^{14}\text{CO}_2$ evolution in water samples accounted for 2.9 and 0.9% of the total applied ^{14}C as glyphosate and metolachlor, respectively (Table 12). Even though mineralization of glyphosate can vary among environmental samples, these values are considerably lower than those reported for soil ecosystems (Accinelli et al., 2005; Getenga and Kengara, 2004; Strange-Hansen et al., 2004). Addition of sediments resulted in an intense increase in glyphosate and metolachlor mineralization. At the end of the incubation period, mineralization of both glyphosate and metholachlor in samples containing 5% sediments was approximately 10 times higher than that observed in water samples. These findings confirmed the important role of microorganisms in glyphosate and metolachlor mineralization. Moreover, the lack of herbicide mineralization in sterilized water suggested that chemical degradation is not a major pathway of degradation of these two chemicals (Table 12). Results from this mineralization experiment reinforced the concept that a major factor limiting a more rapid degradation of OC in surface water of the CER irrigation canal is represented by the low metabolic potential of this ecosystem. Information concerning degradation and other environmental aspects of these two chemicals in surface water is scarce (Tsui and Chu, 2003). Based on the results from a recent monitoring investigation conducted in the USA, Kolpin et al. (2006) speculated that glyphosate would be much more persistent in surface waters than in soil. The presence of a low concentration ($1.5 \mu\text{g mL}^{-1}$) of the antiviral drug OC did not reduce the potential of water from the irrigation canal to mineralize the two studied herbicides (Table 12). These are the first data concerning environmental aspects of the antiviral drug OC or other neuraminidase antivirals. Considering that degradation of glyphosate and metolachlor is to some extent related to the size of indigenous bacteria, these findings suggest that the moderate persistence of OC in water was mainly caused by the low metabolic potential of the water microbial community rather than indirect effects of OC on microorganisms.

	Cumulative $^{14}\text{CO}_2$ evolution			
	^{14}C -Glyphosate		^{14}C -Metolachlor	
	Control	OC treated	Control	OC treated
Water	2.85±0.15	2.91±0.22	0.85±0.07	0.81±0.09
Steril water	0.82±0.06	0.88±0.06	0.11±0.02	0.09±0.02
Water/sediment	38.22±3.98	36.35±4.11	9.91±1.21	8.75±1.39

Table 12– Effect of oseltamivir carboxylate (OC) on $^{14}\text{CO}_2$ evolution from radio-labelled glyphosate and metholachlor within the 36-day incubation period (% of initial radioactivity).

4.1.2 River Po and Venice Lagoon

Chemical analysis

Concentrations of OC in samples of the River Po over the course of the 21-day incubation period are shown in Figure 15. No appreciable degradation of OC was observed in water. In contrast, addition of sediments promoted OC degradation. More specifically, OC degradation in samples containing 5% sediments was adequately described by the first order kinetic model ($r^2 = 0.91$) with a calculated half-life of 15 days. Degradation of OC was not further stimulated by raising the sediment content to 10%.

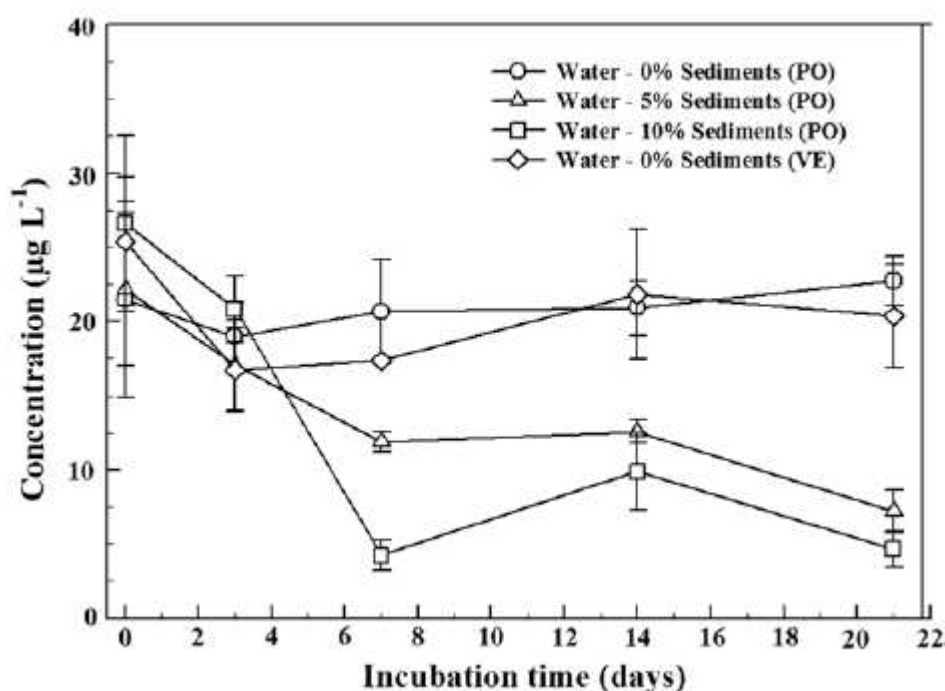


Figure 15 - Degradation of oseltamivir carboxylate (OC) in water and water/sediment samples of the River Po and in water of the Venice Lagoon. Bars represent standard deviations of the means.

These findings are consistent with those of the mineralization study. As indicated in Figure 16, less than 1% of $^{14}\text{CO}_2$ was evolved from water samples. Cumulative $^{14}\text{CO}_2$ evolution was approximately 10-times higher in samples containing 5% of sediments. Addition of a higher amount of sediments did not lead to a significant increase of $^{14}\text{CO}_2$ evolution. Since no appreciable OC mineralization was

observed in sterilized water and water/sediment samples (data not shown), results suggest that the fate of OC in the aquatic ecosystem was governed by microbial processes, thus confirming previous findings on the CER irrigation canal study. The stimulatory effects of sediments on OC degradation were likely due to a greater size of the microbial population of samples containing sediments.

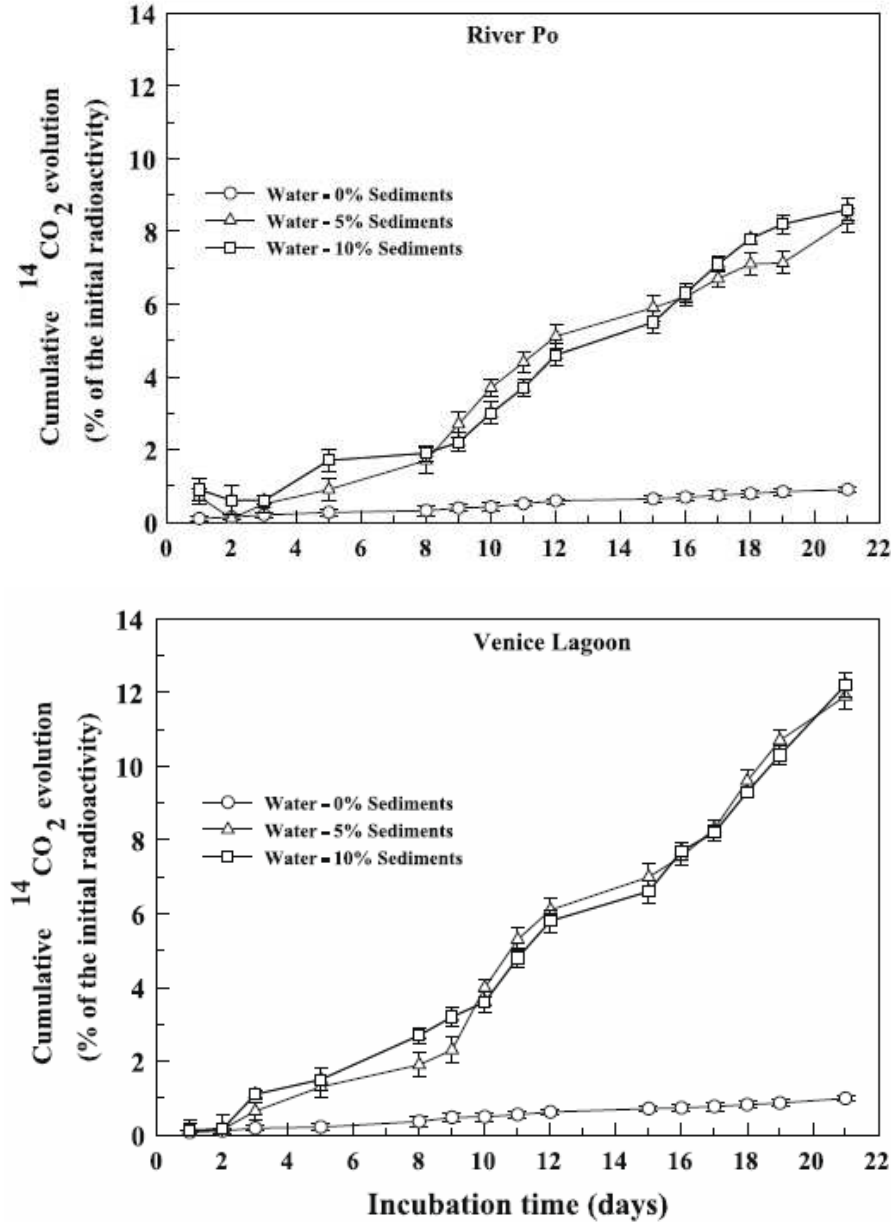


Figure 16 - Cumulative $^{14}\text{CO}_2$ evolution from water and water sediment samples of the River Po and Venice Lagoon. Bars represent standard deviations of the means.

Similar to water of the River Po, OC concentration remained approximately stable in water samples of the Venice Lagoon (Figure 15). In contrast to river samples, concentrations of recovered OC of water and water/sediment samples did not

significantly change over the incubation period (data not shown). Using the extraction method proposed by Fick et al. (2007), the average recovery for OC in water and water/sediment samples of the River Po was >95%. Less OC was recovered from samples of the Venice Lagoon. More specifically, recovery efficiencies were 85 and 47% in water and water/sediment samples, respectively, with no effect of the sediment level. These low recoveries combined with high relative standard deviations did not permit to correctly describe the fate of OC in Venice samples despite the usage of a deuterated internal standard. Mineralization of ^{14}C -OC in Venice samples is shown in Figure 16. At the end of the 21-day incubation, total accumulated $^{14}\text{CO}_2$ from Venice did not exceed 1% of the total applied ^{14}C as OC. As observed with samples of the River Po, addition of sediments led to a significant increase of $^{14}\text{CO}_2$ evolution, with no effect of sediment percentage. Water/sediment samples from Venice Lagoon had a higher potential to mineralize the antiviral drug OC. Since sediments from the two sites had comparable size of the microbial population and distribution of particles size, one possible explanation is the fact that the higher content of organic carbon of Venice sediments would provide more nutrients for sustaining the microbial activity.

Retention and bioavailability of OC in water and water/sediments

Table 13 summarizes the partition of ^{14}C -residues between liquid and solid phase of water samples containing 5% of samples at the end of the 21-day incubation period.

	Liquid phase		Sediments		
	% of the total ^{14}C applied	Total % of the total ^{14}C applied	Bioavailable fraction % of the total ^{14}C remaining in	Sorbed fraction sediments	Bound residues
Po	73.1	16.3	79.3	13.5	4.4
Venice	66.6	20.9	59.8	28.9	7.2

Table 13 – Partition of ^{14}C -residues between liquid phase and sediment of samples of the River Po and Venice Lagoon after 21 days of incubation. Sediments were extracted with 0.1 M CaCl_2 (bioavailable fraction), acetonitrile (sorbed fraction). Total ^{14}C -residues of sediments were determined by combustion.

As expected, most of the ^{14}C -residues were recovered from the liquid phase. Approximately 70 and 65% of ^{14}C -residues remained were recovered from the liquid phase of Po and Venice samples, respectively. The high percentage of water-extractable fractions of ^{14}C -residues recovered from the pellet is consistent with the low affinity of OC to sediment particles. Based on mass balance, 14 and 29% of the remaining ^{14}C residues were extracted by the solvent acetonitrile from Po and Venice pellets, respectively. The higher sorption capacity of Venice sediments was confirmed by the determined sorption coefficients. Sorption isotherms are presented in Figure 17.

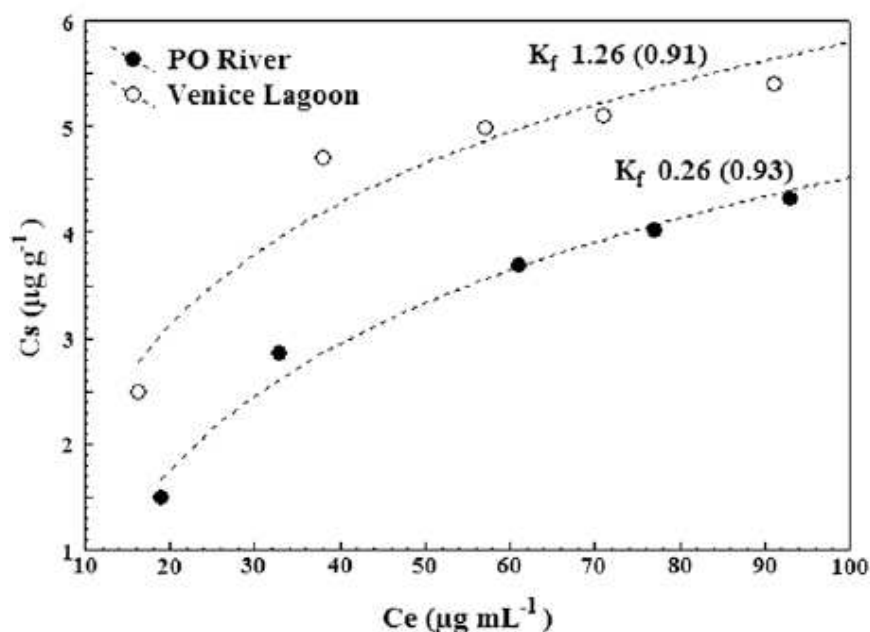


Figure 17 – Sorption isotherms of oseltamivir carboxylate in sediments of the River Po and Venice Lagoon. Calculated Freundlich constant (K_f) and regression coefficients (r^2) are reported.

The Freundlich model adequately described OC sorption to sediments from the two sites ($r^2 > 0.91$). According to several authors, K_f values are directly correlated with sorption capacity of organic xenobiotics (Seybold and Mersie, 1996; Krutz et al., 2004; Accinelli et al., 2006; Sukul et al., 2008). Consequently, the K_f values reported here are compatible with low affinity of OC to sediment particles (Table 13). Sediments from the two sites showed similar particle size distribution (Table 7). Since more organic matter was found in sediments from the Venice Lagoon, the higher K_f values measured in Venice sediments is likely due to its preferential affinity to organic matter. However, other factors than organic matter are expected to influence sorption of OC on sediments.

Microbial analysis

The structure of the bacterial community of Po water was highly variable during the course of the incubation period. As indicated in the dendrogram of genetic distances shown in Figure 18, no clear effects of OC on the diversity of the bacterial community were observed. A relatively high degree of similarity (70%) was only found among samples receiving 0.2 and 2 $\mu\text{g mL}^{-1}$ of OC. Less similarity was observed between samples receiving lower OC dosage (0.02 and 0.002 $\mu\text{g mL}^{-1}$) and the untreated control. Considering the low degree of similarity, results suggest that most of the variability was likely due to other factors than concentration of the antiviral. Similar patterns were observed in water of the Venice Lagoon (data not shown).

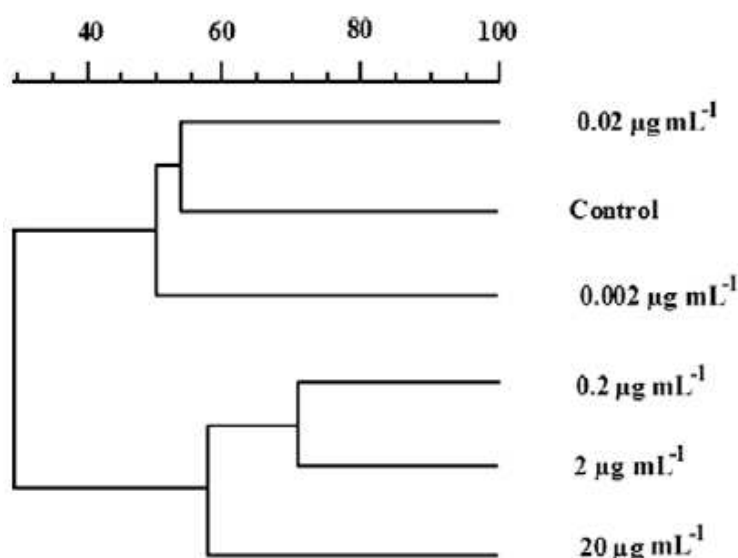


Figure 18 - Dendrogram showing the degree of similarity of ARDRA patterns of Po water samples receiving increasing concentrations of oseltamivir carboxylate and incubated for 10 days.

A number of microbiologically driven processes have been proposed to evaluate the effects of xenobiotics on the aquatic ecosystems (Wagner-Döbler et al., 1992; Moyer et al., 1994; Nazaret et al., 1994; Griebler and Slezak, 2001). Among the different approaches, activity and dynamics of the nitrifying bacterial population have been largely used in environmental studies (Hermansson and Lindgren, 2001; Limpiyakorn et al., 2006; Molina et al., 2007). Nitrification is the biological process of converting ammonia to nitrate via nitrite and is catalyzed by aerobic chemoautotrophic ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing

bacteria (NOB) (Bock et al., 1992). Studies have shown that AOB and NOB are less competitive than the heterotrophic bacteria for oxygen and growing space and are sensitive to environmental inhibition (Van Benthum et al., 1997; Boon et al., 2003; Juliastuti et al., 2003; Limpiyakorn et al., 2004; Pagga et al., 2006). Results from the qRT-PCR analysis showed that OC did not interfere with the expression of three basic genes involved in the bacterial nitrification process (Figure 19).

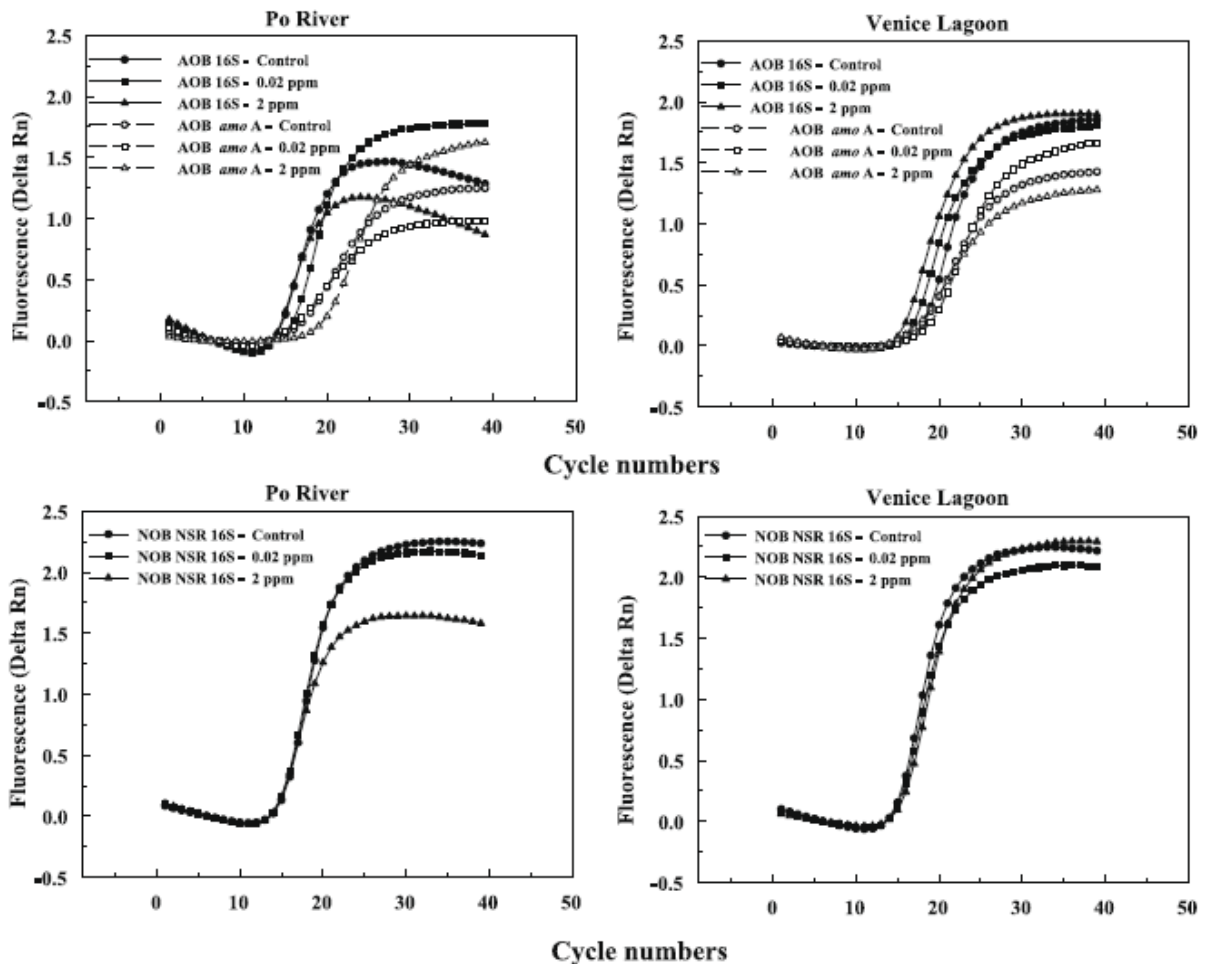


Figure 19 – Representative qRT-PCR analysis of water samples of the River Po and Venice Lagoon incubated for 10 days at 20 °C. Analysis was conducted using primer pairs targeting genes of ammonia-oxidizing bacteria (AOB 16S and AOB *amoA*) and of nitrite-oxidizing bacteria (NOB, NSR 16S).

Using the universal 16S rDNA bacterial primer pairs 1055f/1392r (Ferris et al., 1996), as control references, the present study also showed that increasing concentrations of OC did not affect the transcription process of the highly conserved 16S rDNA. Together, these results confirm that OC would not have detrimental effects on basic bacterial-driven processes of surface water. OC is a selective inhibitor of influenza virus neuraminidase, an enzyme involved in the

release of new virus particles from infected cells. Based on its specific mode of action and in contrast to other pharmaceuticals, which are specifically designed to produce biological effects on bacteria, no direct toxic effect of OC on water microorganisms would be expected. Absence of effects of OC on basic bacterial processes of surface water was also observed in the previous study on the CER irrigation canal. Ecotoxicology studies showed no detrimental effects of OC on other organisms, including the green alga *Pseudokirchneriella subcapitata*, the crustacean *Daphnia magna* and the fish *Danio rerio* (Singer et al., 2008).

4.1.3 Lake Biwa and River Furukawa

Chemical analysis

Degradation patterns of OC in water samples of Lake Biwa and Furukawa river are shown in Figure 20. During the course of the 40 day incubation period, no appreciable decrease of OC concentration was observed in Biwa water. In contrast, samples containing 5% sediments showed an intensification of OC degradation processes. More precisely, the degradation of OC in water/sediments proceeded following the first-order kinetics ($r^2 = 0.97$), with an estimated half-life of 46.2 days. A decrease of OC concentrations over time was also observed in Furukawa samples. Fitting the data to the first-order kinetics yielded half-life values of OC in water and water/sediments of 53.3 ($r^2 = 0.91$) and 38.5 days ($r^2 = 0.96$), respectively. Concentration of OC remained approximately stable in autoclaved samples over the whole incubation period (data not shown). These findings are consistent with the results of the previous studies on irrigation canal CER and on River Po and Venice Lagoon, thus further confirming that the fate of OC in the aquatic ecosystem is driven by microbial process.

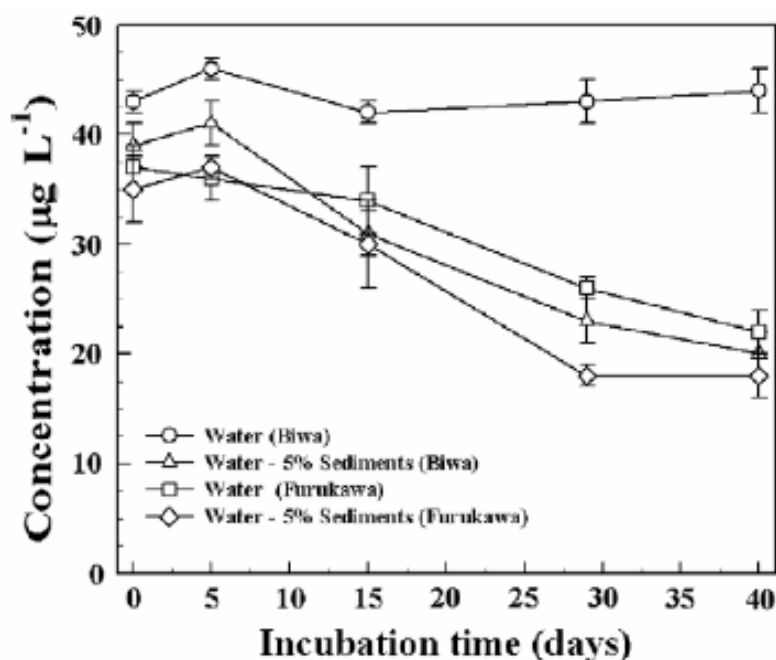


Figure 20 - Degradation of oseltamivir carboxylate in water and water/sediment samples of Lake Biwa and River Furukawa incubated for 40 days. Bars represent standard deviations of the means.

As discussed below, qPCR revealed a large bacterial community in sediments of both Japanese sites. Results of this experiment reinforced the concept that the stimulatory effect of sediments is likely due to the increase of the size of the microbial community. However, this assumption does not support the unexpected rapid degradation of OC in the selected river water. As indicated in Figure 20, degradation of OC in Furukawa water/sediments proceeded after a 2-week long lag phase, thus reflecting the implication of microbial adaptation and/or selection of OC-degrading bacteria. Although no apparent, or a shorter, lag phase was observed in Biwa samples and Furukawa water, the occurrence of adaptation and/or selection processes cannot be excluded in these natural aquatic ecosystems. This explanation is partially supported by the fact that the most promising bacterial strain, which can be used for OC bioremediation programs, was isolated from River Furukawa. In addition, higher nutrient levels of the river would support greater microbial activity than the lake water (Table 8). Using the same procedure described in paragraph d, a preliminary study demonstrated that sediments of River Furukawa had a lower affinity to OC than those of the Lake Biwa (approximately 30% lower; data not shown). The greater bioavailability of OC in Furukawa samples is consistent with the more rapid degradation of the antiviral drug.

OC mineralization

Results of the mineralization study are summarized in Figure 21. Mineralization of OC in water of Lake Biwa, expressed as $^{14}\text{CO}_2$ evolution, accounted for <1% of the initial ^{14}C added as ^{14}C -OC. During the same 40-day incubation period, a higher mineralization was observed in water samples from River Furukawa. Similarly to what was observed in the OC degradation study, the addition of 5% sediments led to an increase of $^{14}\text{CO}_2$ evolution. More precisely, the cumulative mineralization accounted for 12.8% and 21.0% in Biwa and Furukawa samples, respectively (Figure 21). No appreciable $^{14}\text{CO}_2$ evolution was observed in sterile samples (data not shown). Differences of OC mineralization among samples of the two Japanese aquatic environments are compatible with results of the OC degradation study. Negligible mineralization of OC in surface water had been also observed in River Po and Venice samples, and by other authors (Straub, 2009). Slow degradation rates in surface water are commonly reported for a number of xenobiotics, including pesticides (Accinelli et al., 2007).

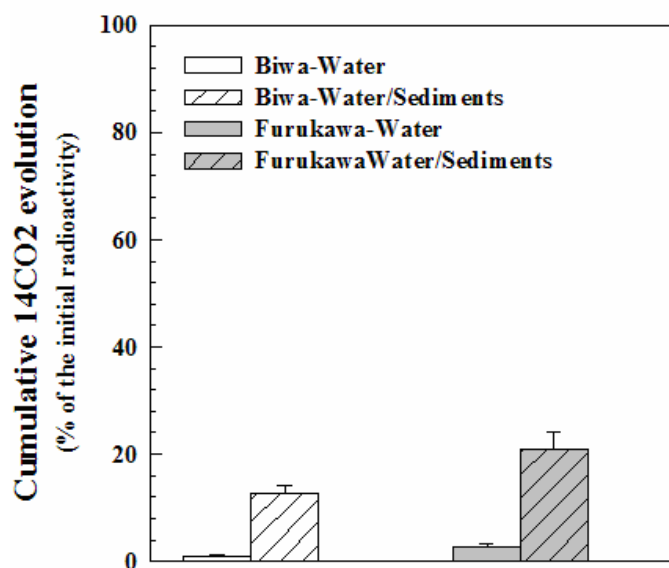


Figure 21 - Cumulative $^{14}\text{CO}_2$ evolution from water and water sediment samples of the Lake Biwa and River Furukawa. Samples were incubated for 40 days. Bars represent standard deviations of the means.

Microbial analysis

Potential effects of the antiviral drug OC on diversity and dynamic of the microbial community of the two selected Japanese waters were estimated using the following DNA-based methods: DGGE, ARDRA and qPCR. DGGE profiles of 16S rDNA fragments amplified from total water and sediments DNA are shown in Figure 22. In both samples, DNA band profiles revealed small differences in term of band numbers and electrophoretic distances. Band profiles were subjected to a numerical analysis based on the Dice similarity coefficient, followed by cluster analysis (Figure 22). The effect of incubation on untreated and OC-treated Furukawa samples was variable, with Dice coefficients ranging from 0.30 to 0.67. Similar differences were observed in Biwa samples, with coefficients ranging from 0.57 to 0.84 in untreated sediments and untreated water, respectively. After 30 days of incubation, cluster analysis revealed that there were no major changes between untreated and OC-treated samples of both sites (Dice similarity coefficients >0.71). These findings are similar to those of the ARDRA (Dice similarity coefficients >0.80; data not shown).

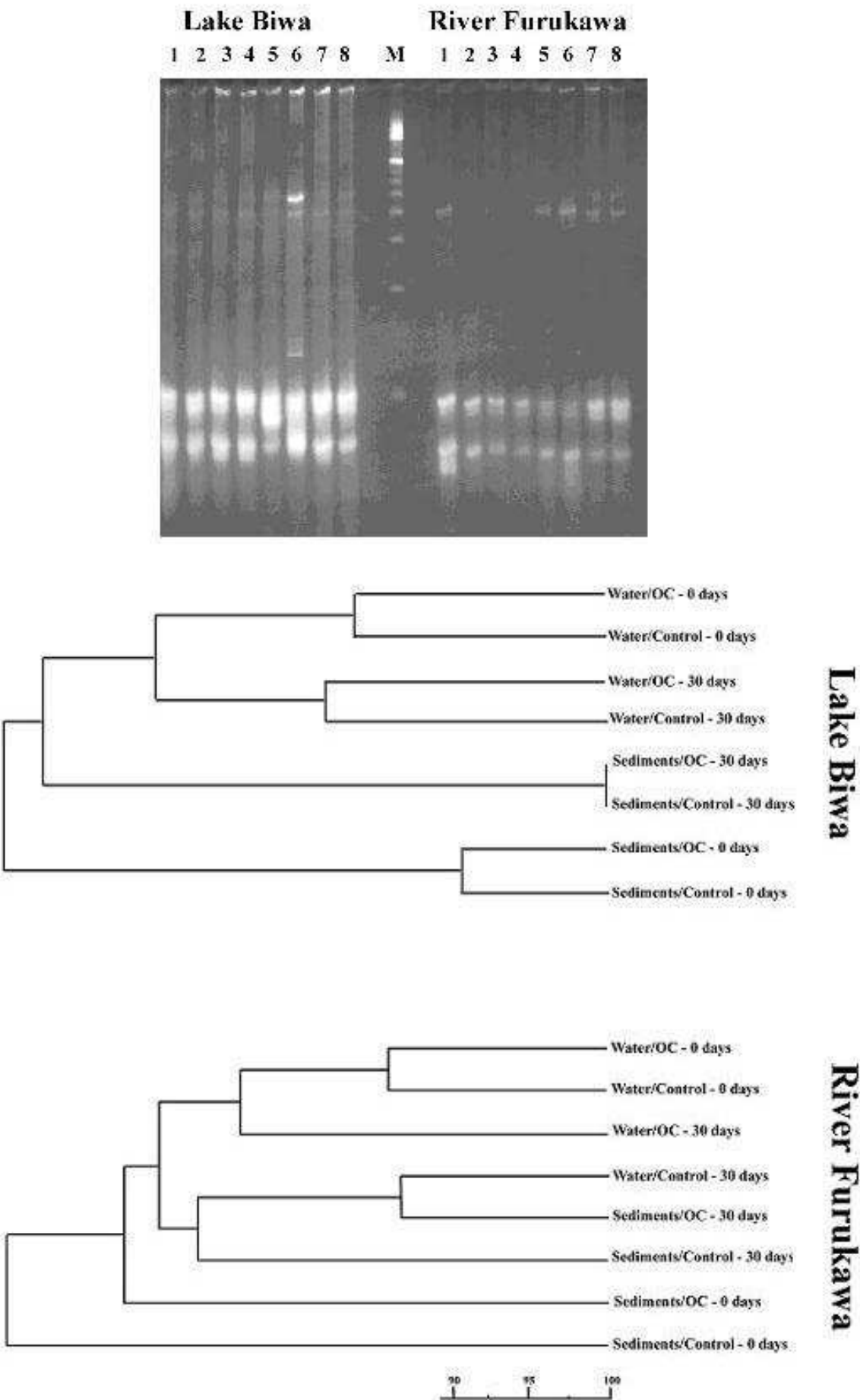


Figure 22 - PCR-DGGE fingerprints (top) and dendrograms of band profiles (bottom) of incubated samples of Lake Biwa and River Furukawa. Lanes 1 and 2: water from OC-treated and untreated samples at 0 days of incubation; lanes 3 and 4: sediment from OC-treated and untreated samples after 0 days of incubation; lanes 5 and 6: water from OC-treated and untreated samples after 30 days of incubation; lanes 7 and 8: sediments from OC-treated and untreated samples after 30 days of incubation; M: DNA marker.

Effects of xenobiotics on the aquatic microbial community depend on a variety of factors, including chemical structure, concentration, their target site, etc. (Brandt et al., 2004; Accinelli et al., 2007). Tamiflu is an inhibitor of the viral neuraminidase or sialidase enzyme (von Itzstein, 2007). Neuraminidases are widespread in animals and microorganisms and catalyze the release of terminal sialic acid residues from glycoconjugates (Taylor, 1996). While the role of bacterial neuraminidases of certain pathogenic bacteria has been elucidated, its significance in the microbial community of water or other environments remains unexplored (Soong et al., 2006). Results presented here demonstrated that concentration of OC up to $40 \mu\text{g L}^{-1}$ has no effects on the structure of the microbial community of the two Japanese aquatic ecosystems. Working with different organisms, including algae, daphnia, fish, and marine algae and invertebrates, Straub (2009) and Hutchinson et al. (2009) have reached the same conclusions. DNA fingerprinting techniques based on total pool of 16S rDNA fragments, including DGGE, ARDRA and other similar methods, are widely used tools for studying the community structure and diversity of microorganisms. In recent years, qPCR has emerged as a promising technique in environmental microbiology (Fierer et al., 2005). More specifically, qPCR-based methods have been largely used to estimate potential effects of xenobiotics on microorganisms (Kim et al., 2007). In this experiment the potential effects of OC on the size of the whole bacterial community and on nitrifying bacteria have been investigated. Results of the qPCR analysis targeting the conservative 16S rDNA, the 16S rDNA of ammonia-oxidizing bacteria and the *amoA* gene involved in the conversion of ammonia to nitrite are presented in Figure 23. OC did not affect copy numbers of the three target genes, thus indicating that the presence of OC did not significantly influence the size of the total bacterial community and of the ammonia-oxidizing bacteria. This is consistent with the expression levels reported in the River Po and Venice Lagoon experiment.

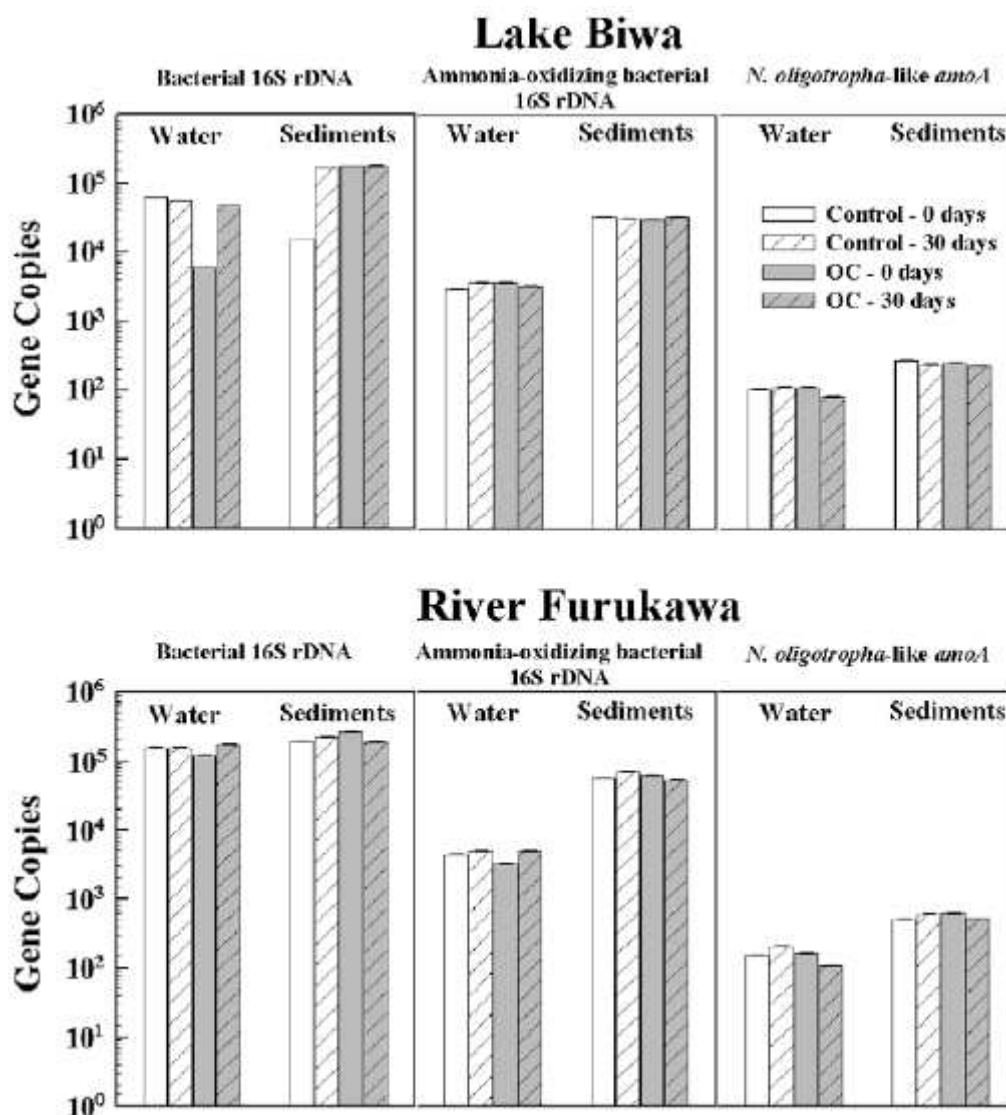


Figure 23 - Quantitative PCR results of Lake Biwa and River Furukawa samples incubated for 30 days. Results are expressed in copy numbers of genes targeting the conservative bacterial 16S rDNA, the ammonia-oxidizing bacterial 16S rDNA and the *Nitrosomonas oligotropha*-like *amoA* gene.

A more practical approach for increasing the removal of OC would be the use of OC-degrading bacteria. In the present study, a basic enrichment culture technique was employed for isolating OC-degrading bacterial strains. After six serial transfers to MSM, two bacterial strains, *Nocardioides* sp. and *Flavobacterium* sp., able to use OC as sole carbon source and energy were isolated from Lake Biwa and River Furukawa, respectively. As shown in Figure 24, the *Flavobacterium* sp. strain isolated from River Furukawa was the most promising for potential use in bioremediation programs. Considering the higher potential of Furukawa water to degrade/mineralize OC, it is not surprising that the more efficient strain was

isolated from this water body. After 10 days of incubation, approximately 6% and 11% of the $^{14}\text{CO}_2$ was evolved in MSM inoculated with the *Flavobacterium* sp. strain and the consortium of the two bacterial strains, respectively (Figure 24).

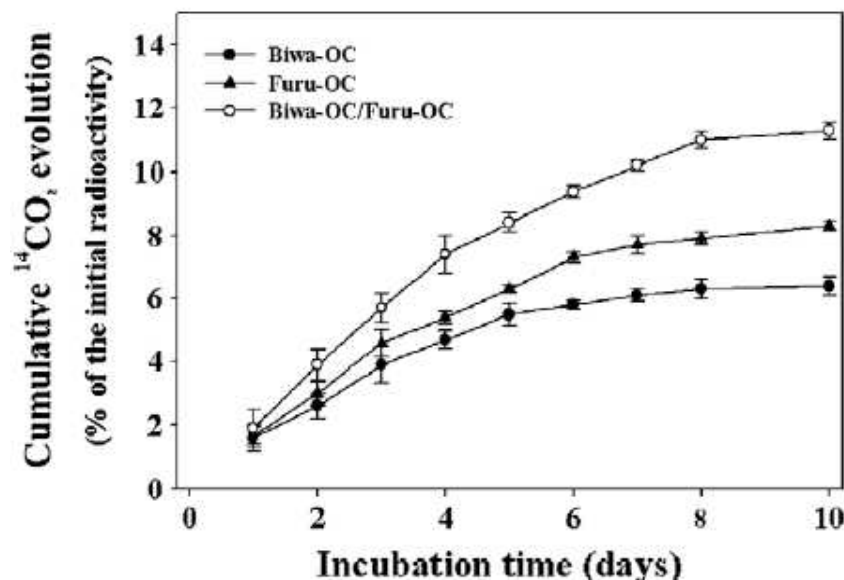


Figure 24 - Patterns of cumulative $^{14}\text{CO}_2$ evolution from a mineral salt medium supplemented with ^{14}C -oseltamivir (OC) as a sole carbon source, and inoculated with OC-degrading bacterial strains isolated from Lake Biwa (Biwa-OC) and River Furukawa (Furu-OC), including a consortium of the two strains. Bars represent standard deviations of the means.

Since we were interested to test the *in situ* ability of the two strains to remove OC, environmental samples were included in the study. Mineralization values of OC in surface water samples inoculated with the OC-degrading strains are reported in Table 14.

	Total evolved $^{14}\text{CO}_2$ (% of the initial radioactivity)			
	Control	Biwa-OC	Furu-OC	Biwa-OC+Furu-OC
Lake Biwa	0.42±0.1	4.77±1.1	7.23±1.9	11.33±2.0
River Furukawa	1.33±0.1	6.31±1.7	10.14±3.0	19.75±1.8

Table 14 - Mineralization of oseltamivir carboxylate (OC) in water samples of Lake Biwa and River Furukawa. Samples were separately inoculated with OC-degrading bacterial strains isolated from Lake Biwa (Biwa-OC) and River Furukawa (Furu-OC), and a consortium of the two strains. Samples were incubated for 10 days. Data are expressed as total $^{14}\text{CO}_2 \pm$ standard deviation. Control consists in uninoculated water samples.

4.1.4 Wastewater Treatment Plant of Bologna

Chemical analysis

In this study, we were also interested to investigate the fate of OC in samples representing two different steps of the ordinary wastewater treatment process. Consequently, we investigated the mineralization of OC in samples collected from the WWTP of Bologna. In particular, OC mineralization was evaluated in samples of ASML and EW. Cumulative $^{14}\text{CO}_2$ evolution from ASML and EW samples over the course of the 40-day incubation period are reported in Figure 25. ASML showed a high potential to mineralize OC. Approximately 75% of the initial radiolabeled OC evolved as $^{14}\text{CO}_2$ during the 40-day incubation period. In contrast, OC mineralization in EW accounted for <37%.

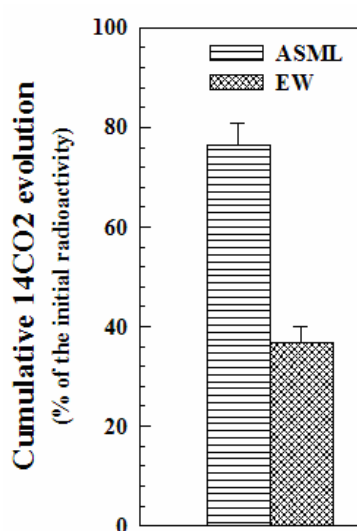


Figure 25 - Cumulative $^{14}\text{CO}_2$ evolution from water and water sediment samples of the wastewater treatment plant of Bologna, including activated-sludge-mixed liquor (ASML) and effluent water (EW). Samples were incubated for 30 days. Bars represent standard deviations of the means.

Among other factors influencing mineralization of xenobiotics (i.e. bioavailability, etc.), the higher metabolic potential of ASML with respect to EW is likely explained by differences in the size of the microbial population and nutrient content. The WWTP of Bologna is designed to remove approximately 80% of the BOD and total suspended solids entering the plant. In addition, it should be also considered that wastewater effluents are treated with sodium hypochlorite before discharging in the canal. Obviously, the disinfection step is expected to reduce the microbial activity of EW and thus its potential to

mineralize the antiviral drug OC. In a laboratory study, simulating normal sewage treatment, Fick et al. (2007) demonstrated that OC is not removed during the entire process. Similar conclusions are reported by Straub (2009). A common aspect of these studies is that incubation period of OC-treated wastewater samples did not exceed 24 hours. The main reason we decided to investigate the mineralization of OC in ASML and EW was to verify the potential application of bioremediation approaches directly in WWTPs. This information can be useful for setting up practical strategies for the removal of OC during the wastewater treatment process. Obviously, the long incubation period chosen for this study is not compatible with the shorter hydraulic retention times commonly adopted in ordinary WWTPs.

Microbial analysis

The *in situ* ability of the two isolated strains to remove OC was tested by measuring OC mineralization in ASML and EW (Table 15). The superior ability of the consortium was observed in wastewater samples. However, less differences among inoculated and uninoculated samples were observed in EW and especially in ASML.

	Total evolved $^{14}\text{CO}_2$ (% of the initial radioactivity)			
	Control	Biwa-OC	Furu-OC	Biwa-OC+Furu-OC
Bologna ASML	29.14±2.8	36.99±3.8	39.72±4.1	42.53±4.8
Bologna EW	12.11±1.6	37.68±6.0	39.11±4.4	49.45±6.2

Table 15 - Mineralization of oseltamivir carboxylate (OC) in water samples of the wastewater treatment plant of Bologna, including activated-sludge mixed liquor (ASML) and effluent water (EW). Samples were separately inoculated with OC-degrading bacterial strains isolated from Lake Biwa (Biwa-OC) and River Furukawa (Furu-OC), and a consortium of the two strains. Samples were incubated for 10 days. Data are expressed as total $^{14}\text{CO}_2 \pm$ standard deviation. Control consists in uninoculated water samples.

These findings are compatible with the larger microbial population of these samples and consequently with the higher selective pressure on the introduced strains. In addition, the superior ability of *Flavobacterium* sp. to metabolize xenobiotics when inoculated with other bacteria has been also observed by Kawai and Yamanaka (1986).

Bioremediation assay using a *P. chrysosporium* formulate

The Patent Pending bioremediation formulation of the white rot fungus *P. chrysosporium*, was assayed for its ability to remove the antiviral drug OC from samples of the WWTP. Decrease of OC concentrations proceeded exponentially in ASML, with no evident lag phase (Figure 26). A significantly higher ($P < 0.05$) amount of OC was removed from samples amended with the bioremediation formulation. Decrease of OC concentration in control and bioremediated samples obeyed to the first-order kinetic model ($r^2 > 0.9$), with estimated half-lives of 69 and 17 days, respectively.

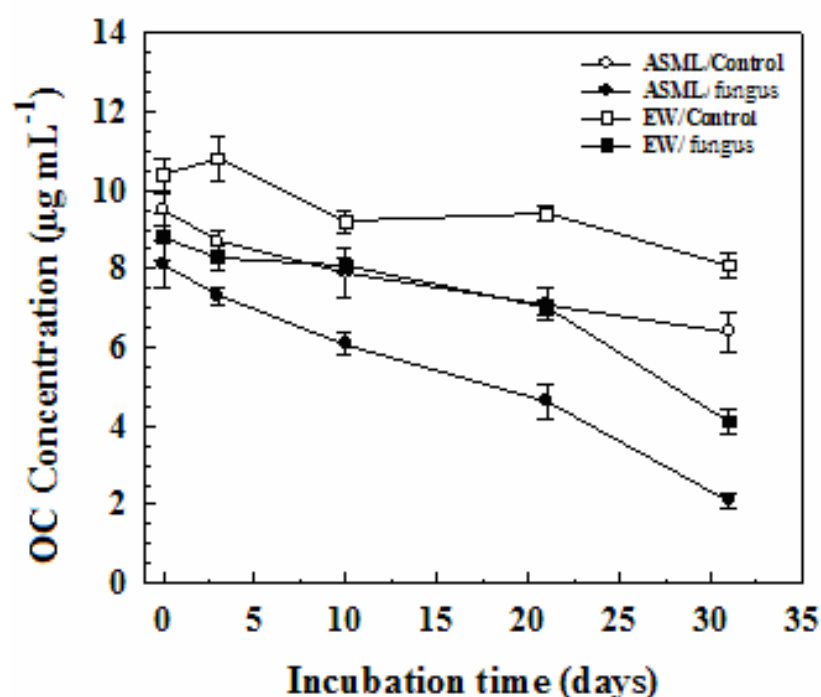


Figure 26 - Effect of the fungal formulation (fungus) on degradation of oseltamivir carboxylate in samples of activated-sludge-mixed liquor (ASML) and effluent water (EW). Samples were incubated for 30 days at 20 °C. Bars represent standard deviations of the means.

These findings are compatible with high efficiency of the formulation in the removal of OC from ASML. In addition, results of the present experiment further support the ability of *P. chrysosporium* to degrade a large list of different compounds, now including the recently introduced antiviral drug OC. The capability of this WRF to degrade OC and, more generally, antiviral drugs have not been reported in the literature. Considering the importance of OC for treating regular seasonal flu, its potential use in the case of pandemic flu scenarios (Singer

et al., 2008), the proposed formulation would represent a practical alternative for future bioremediation programs.

Degradation of OC in EW (Figure 26) did not fit the first-order kinetic model. However, and as discussed below, even considering that OC was more persistent in EW, removal of OC was significantly stimulated ($P < 0.05$) in bioremediated samples.

After 30 days of incubation, the cumulative evolution of $^{14}\text{CO}_2$ accounted for 40 and 69% of the initial radioactivity in control and bioremediated ASML samples, respectively (Figure 27). Less $^{14}\text{CO}_2$ evolved from EW samples (Figure 27). These findings are consistent with the previous experiments, that demonstrated that OC degradation and mineralization in water and wastewater is mainly driven by microbial process. Before entering the receiving canal, water from the WWTP is subjected to microfiltration and chlorine oxidation treatments. In addition to microbial oxidation occurring in the biological tank, these two latter treatments, are expected to further reduce the microbial activity of EW.

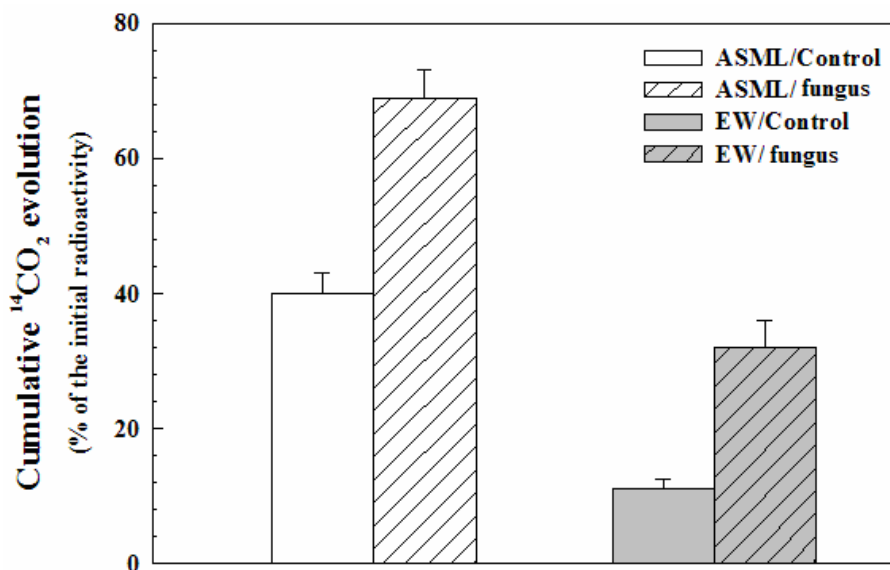


Figure 27 - Effect of the fungal formulation (fungus) on cumulative $^{14}\text{CO}_2$ evolution of oseltamivir carboxylate from samples of activated-sludge-mixed liquor (ASML) and effluent water (EW). Samples were incubated for 30 days at 20 °C. Bars represent standard deviations of the means.

Analysis of the bacterial 16S rDNA PCR products by DGGE (Figure 29) indicated the structure of the microbial community of ASML and EW over the incubation period. The introduced formulation did not cause major changes in the

bacterial community diversity. And as expected, high degree of similarity was observed within bacterial populations in OC-treated and non-treated samples, thus confirming the low impact of OC on indigenous bacteria populations.

4.2 CHAPTER 2 – Common use antibiotics in WWTP

Two of the three antibiotics, sulfamethoxazole and erythromycin were rapidly degraded in ASML (Figure 28).

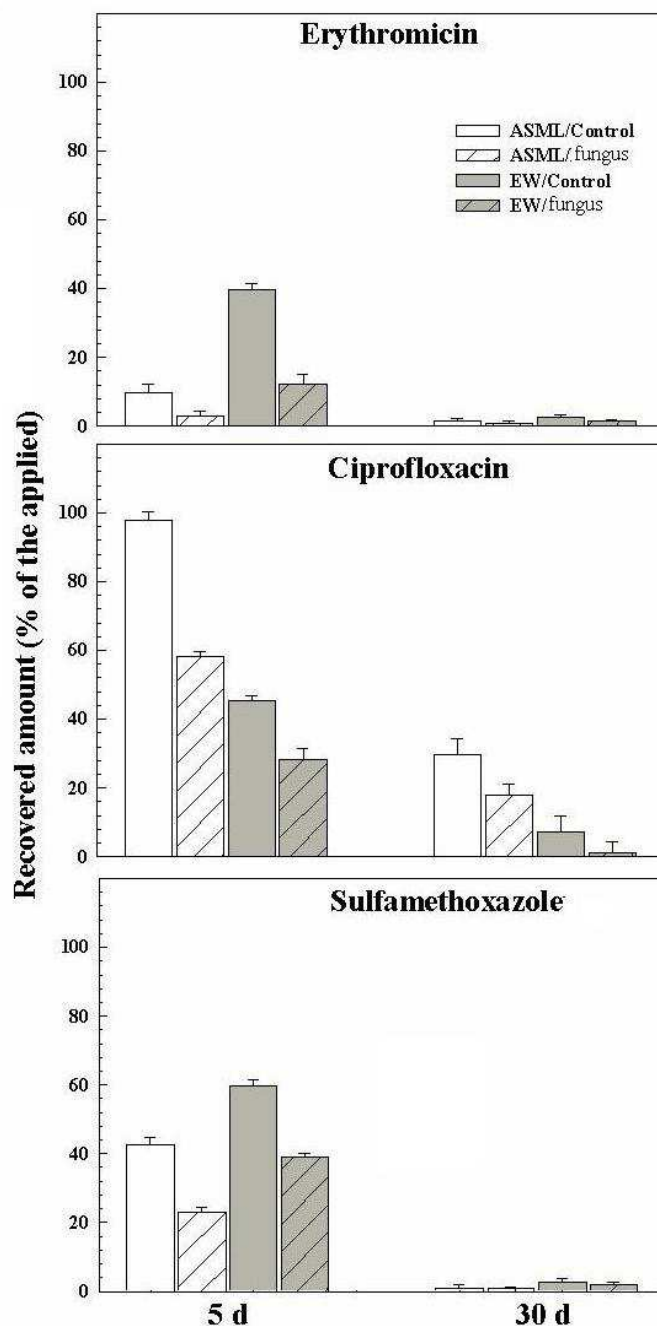


Figure 28 – Efficiency of the formulation (fungus) in the removal of erythromycin, sulfamethoxazole and ciprofloxacin from samples of activated-sludge-mixed liquor (ASML) and effluent water (EW). Data are expressed as percentage of recovered active substance after 5 and 30 days of incubation. Bars represent standard deviations of the means.

The effect of the bioremediation formulation was clearly distinguishable after 5 days of incubation in both ASML and EW samples. In the case of the antibiotic ciprofloxacin, this positive effect was also distinguishable at the end of the incubation period. In particular, after 30 days of incubation, 30 and 18% of ciprofloxacin were removed from bioremediated and control ASML samples, respectively. In contrast to the other pharmaceuticals, more ciprofloxacin was removed in EW than in ASML, with a significant stimulatory effect of the formulation. The results of this experiment demonstrated the efficacy of the bioremediation approach.

PCR amplification of the conservative bacterial 16S rDNA produced fragments of the expected size (data not shown). Separation of PCR products by DGGE resulted in distinct band profiles (Figure 29). Analysis of DGGE profiles indicated that the structure of the microbial community of ASML and EW changed over the incubation period. Band profiles of samples receiving the same pharmaceutical were generally similar, thus suggesting that major changes were caused by pharmaceuticals rather than the introduced formulation. Similarity within the bacterial community was less pronounced at the end of the 30-day incubation period.

4.3 CHAPTER 3 – Veterinary pharmaceuticals

Analysis of bacterial community composition by FISH

The structure of the autochthonous bacterial community of the soil incubated with oxbendazole and doramectin was determined at the phylogenetic level by Fluorescence *In Situ* Hybridization.

The 21-day incubation of soil with $5 \mu\text{g mL}^{-1}$ of oxbendazole (Figure 30) determined significant changes in the *Archaea*, *Cytophaga-Flavobacterium* and *Firmicutes*-LGC taxa, appearing to promote their abundance, in respect to the non-incubated control. Among the remaining groups, only the ϵ -*proteobacteria* were significantly depressed by the presence of oxbendazole, and the main phylogenetic groups of the *Proteobacteria* did not seem to be affected. These findings suggest that, those of the bacterial groups that are promoted by the drug, might be involved in its degradation, and that the presence of a low concentration of oxbendazole does not have any detrimental effects on the overall bacterial community phylogenetic structure.

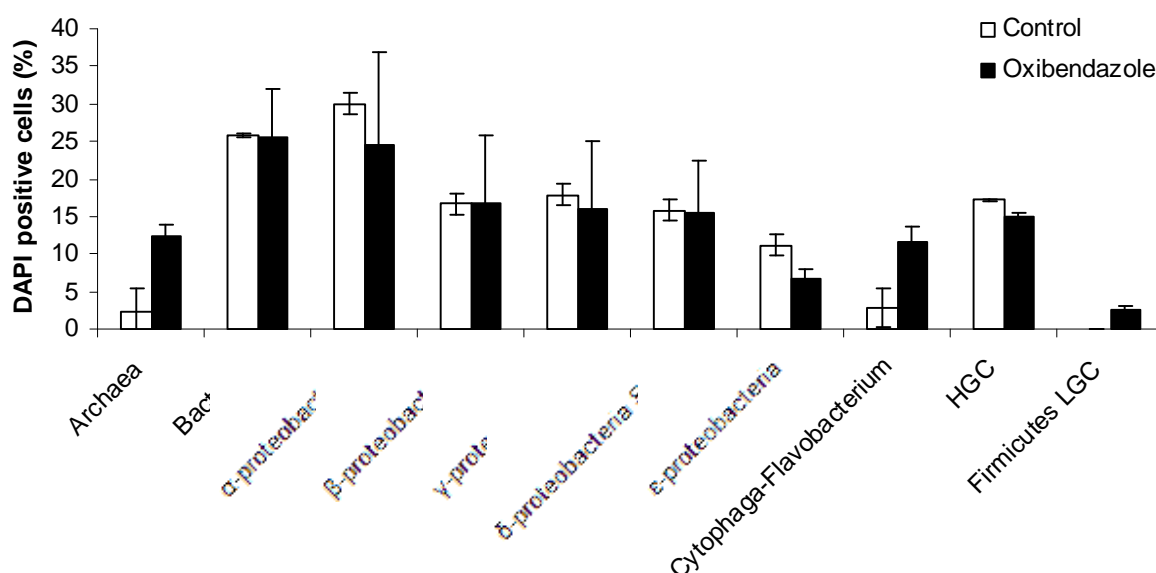


Figure 30 – Bacterial community structure detected by FISH, in soil incubated for 21 days with $5 \mu\text{g mL}^{-1}$ of oxbendazole and control. The values are expressed as % of the DAPI positive cells, and are means of four analyses. Vertical bars represent standard errors.

The presence of $20 \mu\text{g mL}^{-1}$ of doramectin affected the bacterial community phylogenetic structure by depressing significantly ($p < 0.01$) the *Bacteria* and α -, γ -, ϵ -, δ -*proteobacteria* groups (Figure 31). On the contrary the β -*proteobacteria* cluster seems to be enhanced by the presence of the veterinary drug in soil.

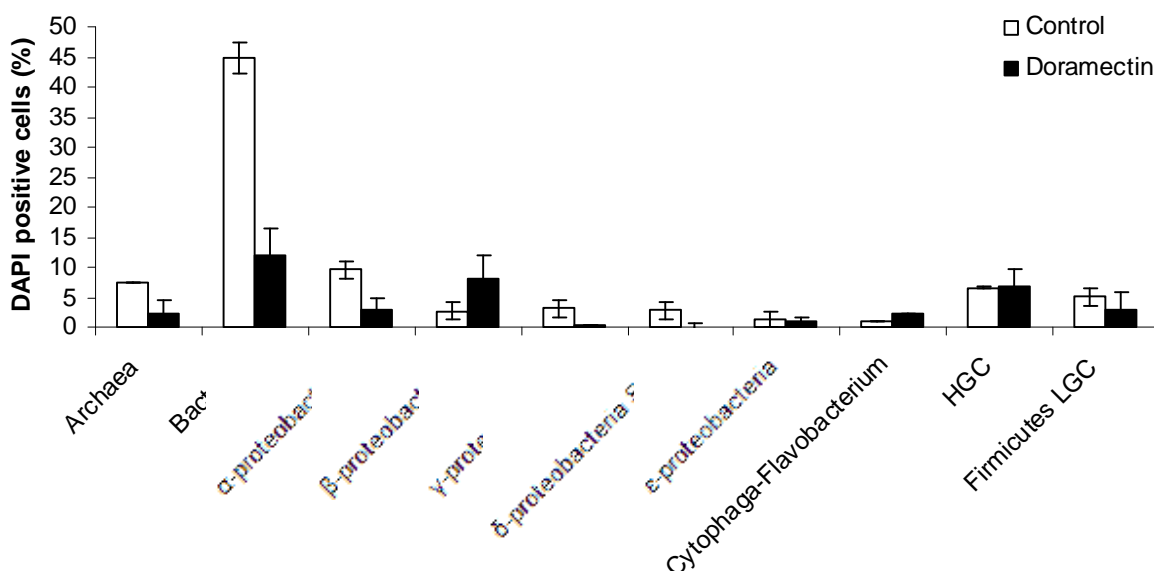


Figure 31 – Bacterial community structure detected by FISH, in soil incubated for 23 days with $20 \mu\text{g mL}^{-1}$ of doramectin and control. The values are expressed as % of the DAPI positive cells, and are means of four analyses. Vertical bars represent standard errors.

Furthermore FISH analysis, using the *narG* probe, permitted the gene detection in incubated and non-incubated soil samples. The presence of both, oxibendazole and doramectin caused a significant ($p < 0.05$) increase in the *narG* positive % of the DAPI stained cells (Table 16) in comparison to the non-incubated control. This result suggests that the bacterial community might use the chemical drug as a substrate for metabolism, possibly involving its degradation.

<i>narG</i> positive cells (%DAPI)	
Control	4.64 ± 2.58
Oxibendazole	11.70 ± 9.88
Control	1.04 ± 1.23
Doramectin	22.73 ± 17.23

Table 16 – Number of *narG* positive cells (% DAPI) in incubated soils and control, analyzed by FISH. Values are the means of four replicates \pm standard deviations.

Analysis of *narG* gene by PCR and qRT-PCR

The presence and expression level of the *narG* gene in soil samples, incubated with the veterinary drugs and controls, was further confirmed by classical PCR amplification, from extracted DNA in all the samples, indicating that the gene was ubiquitous. The change in expression of the *narG* gene in the sample was evaluated by comparison to the control (no treatment) as a ratio, using the 16S gene as a reference. The presence of oxibendazole, did not affect the expression of the *narG* gene, that remained constant in the incubated soils and in the control. This is in line with FISH analysis of the bacterial community that revealed no specific negative effects. On the contrary the presence of doramectin caused a significant increase in *narG*/16S mRNA ratios, from 12 to 55 times. The increase of the *narG*/16S mRNA ratio is possibly due to the global decrease of 16S mRNA, while the portion of remaining bacteria contribute to the denitrification process.

Isolation of bacterial strains

Eleven different bacterial isolates were obtained from the oxibendazole-treated microcosms, and classified as *Bacillus pumilus*, *Bacillus implex*, *Bacillus nealsonii*, *Bacillus thuringiensis*, *Bacillus benzoovorans*, *Bacillus anthracis*, *Acetobacter pasteurianus*, *Bacillus cereus*, *Arthrobacter crystallopoietes*, *Rhodococcus rhodochrous*, and *Klebsiella planticola*. Two different strains were obtained from doramectin-treated microcosms, classified as *Bacillus subtilis* and *Bacillus megaterium*. Strains were tested for their degrading capabilities and qRT-PCR was performed to monitor the expression of *narG* gene in presence/absence of the two veterinary drugs (work in progress, data not shown).

5. CONCLUSIONS

5.1 CHAPTER 1

The first study on CER irrigation canal samples showed the important role played by microbial processes in the degradation of the antiviral drug oseltamivir carboxylate (OC) in surface water. Even though the low amount of $1.5 \mu\text{g mL}^{-1}$ of OC was lost from surface water samples, the potential of OC degradation was significantly greater in water samples containing sediments.

For better understanding the degradation of OC in other environmental scenarios and to elucidate potential effects of this antiviral drug on the structure of the microbial community of surface water, a second laboratory study was conducted on two different aquatic environments located in northern Italy, the River Po and the Venice Lagoon. This study demonstrated the potential of the antiviral to persist in surface water, showing also that in both river and saline water, rapid OC removal can be achieved by addition of a low amount (5%) of sediments to water, which promotes microbial degradation processes. Since OC showed a low sorption affinity to sediments, no reduction of bioavailability is expected after stimulating microbial processes by addition of sediments to environmental samples. Concentrations of OC up to $20 \mu\text{g mL}^{-1}$ did not affect the structure of the microbial community and bacterial nitrification processes. Considering that environmental concentrations of OC in highly populated catchments during an influenza pandemic are predicted to be in excess of $20 \mu\text{g mL}^{-1}$, no detrimental effects of OC on the microbial community are expected.

Further laboratory experiments conducted using samples from the Japanese surface water and sediments of Lake Biwa and River Furukawa, and wastewater from the treatment plant of the city of Bologna indicated that OC degradation is variable and not easily predictable. Major factors influencing the degradation rate of this antiviral drug include intrinsic environmental properties and most importantly the size of the indigenous microbial community. Although slow biotransformation rates were observed in surface water, degradation of OC proceeded more rapidly in wastewater. This suggests that bioremediation techniques would be more successful when applied to wastewater and effluent.

Using different DNA-based approaches, it may be concluded that OC would not affect the structure of the microbial community and the size of the whole bacterial

community, including the group of ammonium-oxidizing bacteria. In the present study the potential use of OC-degrading bacteria was explored for its removal from water and wastewater. Two bacterial strains able to grow using OC as sole carbon and energy sources were isolated. Inoculation of environmental samples with these two bacterial strains demonstrated the feasibility of this technology, especially in surface water and effluents of a wastewater treatment plant.

Furthermore the tested bioremediation strategy using a formulation with the fungus *P. chrysosporium* showed that after 30 days of incubation, the formulation removed more than half the initial amount of OC in both activated-sludge-mixed liquor (ASML) and effluents (EW).

Taken together, these information suggest that prolonged residential time of OC in the biological degradation step of wastewater treatment plants, and the bioremediation approach can be useful strategies for reducing the risk of OC to enter the aquatic ecosystem.

5.2 CHAPTER 2

The bioremediation formulation of *P. chrysosporium* was capable to remove efficiently also three different antibiotics from wastewater, including samples of ASML and EW. In the case of the less persistent pharmaceutical, the antibiotic erythromycin, almost 80% of the applied amount was removed in bioremediated ASML and EW samples within only 5 days of incubation.

5.3 CHAPTER 3

The presence of two veterinary pharmaceuticals, doramectin and oxibendazole, in an agricultural soil affected the overall structure of the bacterial community, analyzed by FISH promoting some phylogenetic groups rather than depressing others. The two drugs were possibly used as a substrate for bacterial metabolism, as suggested by the increase in the *narG* positive cells. Furthermore the isolation of a number of bacterial strains capable to grow on oxibendazole and doramectin was promising for the development of further bioremediation strategies.

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